EXPERIMENTAL CELL RESEARCH

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ACTION OF PERIODATE ON THE SURFACE REACTIONS ATTENDING THE ACTIVATION OF THE EGG OF THE SEA URCHIN, PSAMMECHINUS MILIARIS

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I. PROBLEM, MATERIAL AND METHODS

It was previously shown (9, 13) that dissolved jelly coat substance (jelly solution) from sea urchin eggs inhibits a normal differentiation of the fertilization membrane in these eggs. The membranes remained low, thin and folded. The cortical material did not merge as normally with the vitelline membrane. The effect was interpreted as being due in the first place to an inhibition of the normally occurring coagulation of the vitelline membrane. The jelly solution exerts its effect directly on the egg surface, as the same effect was demonstrable also in artificial membrane formation following an exposure to a hypertonic medium (8). In the summer of 1949 these writers continued the studies on the effect of jelly solution on the surface of the egg of *Psammechinus miliaris* from the neighbourhood of Kristineberg, the Zoological Station of the Swedish Academy of Science. This species proved to be very sensitive to the action of the jelly solution — in this respect it recalls *Arbacia lixula* (13). Certain new features of the action were observed. Oxidation of the jelly substance with periodate removed its inhibitory effect on the surface changes attending activation of the egg. This lead to further observations on the direct effect of periodate on the egg surface, which are also recorded below.

The jelly solution was prepared from heavy egg suspensions subjected to treatment with sea water of pH about 5.4. The jelly substance went into solution at this pH. The eggs were centrifuged off and the jelly solutions stored in frozen state at —20°. Before use they were thawed, diluted with sea water and neutralized to pH 8.0–8.1. After dialysis against distilled

water the stock solution used throughout the experiments had a dry matter content of 0.043 per cent (average of three determinations).

Sodium periodate 0.1 N solution in distilled water was diluted 100 times with sea water, pH 8.0. In this solution the periodate is not stable but is converted to disodiumorthoperiodate, $Na_2H_3I\ O_6$ (6) which soon precipitates from the solution. Before this had occurred 0.1–0.2 ml of the 0.001 N

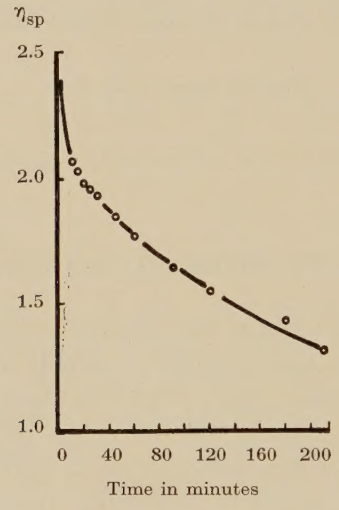


Fig. 1. Specific viscosity, N_{sp} (i.e. viscosity relative to water —1), against time of a 0.045 per cent solution of dissolved jelly coat substance. Temperature 35°.

periodate solution was added to 2 ml jelly solution or egg suspension, the final concentration thus being $5–10 \times 10^{-5}$ N. The decrease in viscosity of a jelly solution which occurs upon addition of the periodate, see Fig. 1, proves that this oxydant was active even at the relatively high pH prevailing in these experiments. A partial conversion of the periodate may have occurred. The concentrations indicated may therefore not be accurate but rather represent a maximum value. Increasing the amount of periodate added to a solution or suspension beyond 0.1–0.2 ml hardly gives any increased effect, due to a partial conversion of the periodate. The solutions and egg suspensions were incubated for some time with periodate, although some experiments showed that this agent acts very rapidly. These writers are aware that the present work merely gives a first orientation concerning the problems under examination.

II. EFFECT OF THE JELLY SOLUTION AND REMOVAL OF THIS EFFECT BY PERIODATE

One typical experiment is described in Table I. Some further data and comments have to be added. In eggs of sample 2, the extruded cortical material was present in the form of globules, spherical units including a number of cortical granules (7, 9, p. 275). In general the conversion of the cortical granules into rods was arrested. In this respect conditions were similar to those previously recorded for the eggs of *Arbacia lixula* (9, p. 278, 13). The membranes were folded and the surface of the eggs appeared wrinkled, but no temporary contraction of the eggs occurred as in samples 1 and 3–5. In sample 2 certain connecting strands extended between membrane and egg surface.

The above as well as reports referring to *Arbacia lixula* eggs (13) convey the impression that the temporary contraction is a prerequisite for the full development of the fertilization membrane. The contraction probably involves an expression from the egg of one or several substances which are instrumental in the coagulation of the membrane (13). The high percentage of di- or plurispermic eggs in sample 2 is obvious. The difference (D) between 2 and 4, 68 per cent, is statistically highly significant ($s = 9.3$, $\frac{D}{s} = 7.3$). In sample 1 no di- or plurispermy occurred. A mere inspection of 3 and 5 made sure that conditions were similar to those prevailing in sample 4. Also in later stages — after about 20 hours' development — striking differences prevailed when sample 2 was compared with samples 1, 4 and 5.

Further quantitative data are given in Table II concerning the di- or plurispermy caused by the addition of jelly solution. The data of this table leave no doubt concerning the significance of the differences between the control in sea water and the samples containing jelly solution. Furthermore the number of spermatozoa as well as the concentration of the jelly solution has varied. Apparently the number of di- or plurispermic eggs increases with increasing number of spermatozoa. More material is, however, required to carry out detailed study of this relationship, see the statistical data laid down in Table II. Conversely, the differences obtained between the control and the different concentrations of jelly solution are highly significant.

Another important result was obtained from this experiment. In a previous note (8) it was shown that jelly substance acts directly on the egg surface. This result is substantiated by observations on the material of Table II. Would

TABLE I

Influence of jelly solution and jelly solution treated with periodate on the fertilization. The eggs were added to samples 1—5 after some minutes and remained thereafter in an unfertilized state for about an hour. Sperm were then added in 5 minutes intervals to samples 1—5.

	1	2	3	4	5
Sea water	2.0	1.5	2.0	1.5	1.5
Jelly solution 0.043 per cent	—	0.5	—	0.5	0.5
Na-periodate 0.001 M	—	—	0.1	0.1	0.2
Observations immediately after insemination of the egg.	Membrane elevation begins 50″ after insemination. A certain contraction of the egg surface which soon regresses. Smooth well elevated membranes.	Low, thin membranes begin to appear 60″ after insemination. No contraction of the egg surface occurs. Membranes remain low and somewhat folded.	Membrane elevation begins 45″ after insemination. Contraction as in sample 1. Smooth and well elevated membranes. Spermatozoa exhibit an increasing tail agglutination.	Membrane elevation begins 75″ after insemination. Contraction more pronounced than in 1. Smooth and well elevated membranes. Single non fertilized eggs.	Membrane elevation begins 45″ after insemination. Contraction as in sample 4. Smooth well elevated membrane. Single non fertilized eggs.
Observations 30—40 min. after insemin.	See Fig. 2.	See Fig. 3.			
Percentage di- or pluri-spermic fertilization. According to observations 50—60 min. after insemin.	—	75 Number counted: 119. See Fig. 4.	Not examined in this respect.	7 Number counted: 93.	Not examined in this respect.
Development after about 20 hours.	Swimming blastulæ with primary mesenchyme. A smaller percentage with increased number of cells in the blastocoel.	> 90 per cent pathological blastulæ with irregular walls. The blastocoel filled with cells. A small number rather normal blastulæ. Increased number of cells in the mesenchyme even here, Fig. 5.	Almost all eggs sticking to the bottom of the glass dish. As a consequence the eggs had undergone abnormal development. Some few swimming blastulæ like those in sample 1.	Swimming normal blastulæ with primary mesenchyme. A small number pathological with increased number of cells in the blastocoel. Development as in sample 1, Fig. 6.	Development as in sample 4.

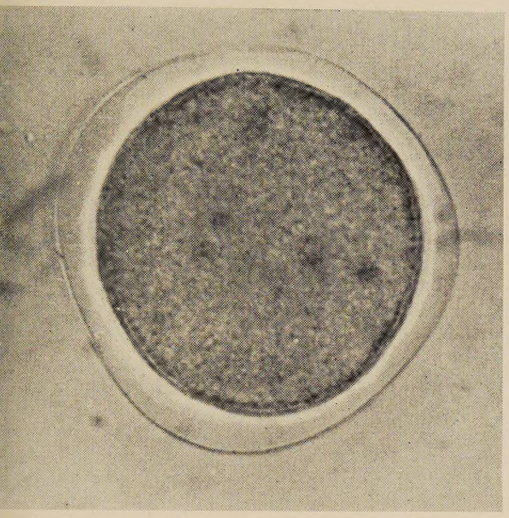


Fig. 2.

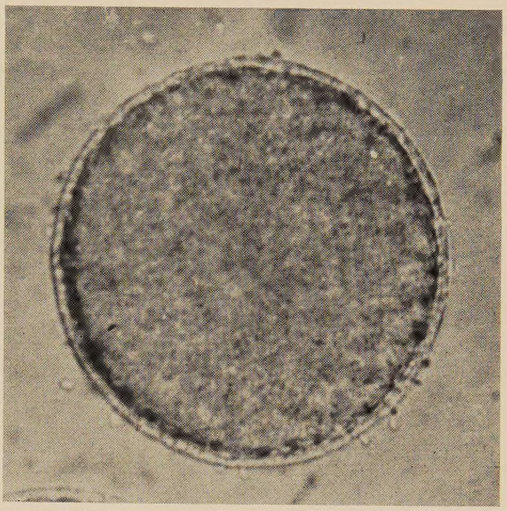


Fig. 3.

Fig. 4.

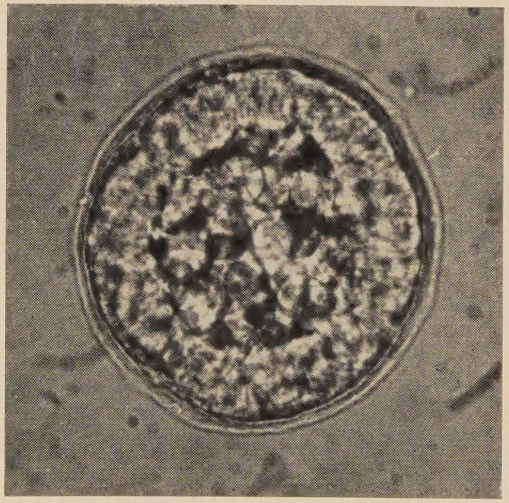
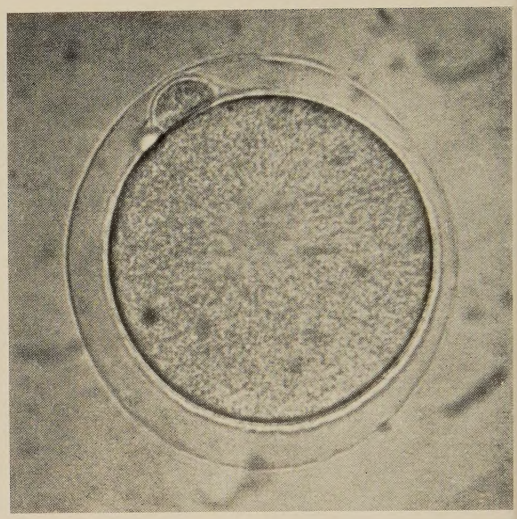


Fig. 5.

Fig. 2. Control egg fertilized in sea water; see Table I, column 1.
Fig. 3. Egg fertilized in presence of dissolved jelly coat substance; see Table I, column 2.
Fig. 4. Egg fertilized in presence of dissolved jelly coat substance, plurispermic eggs; see Table I, column 2.
Fig. 5. Blastula from eggs fertilized in presence of dissolved jelly coat substance; see Table I, column 2.

Fig. 6.



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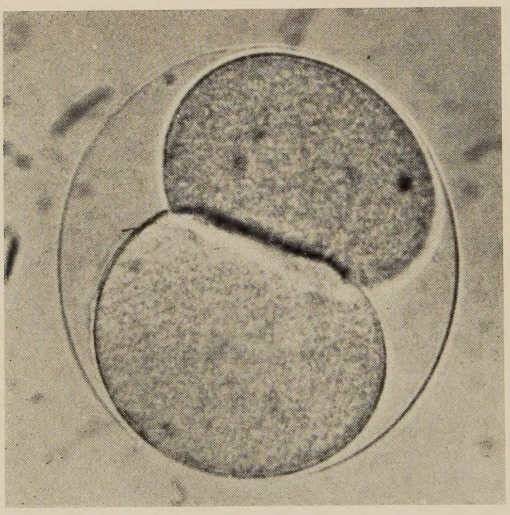

Fig. 8.

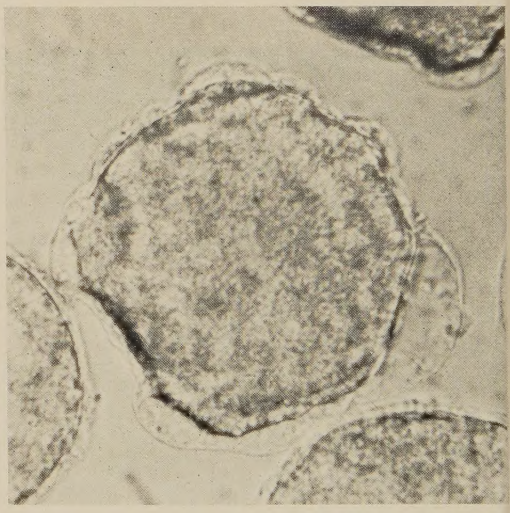

Fig. 9.

Fig. 6. Blastula from eggs fertilized in presence of dissolved jelly coat substance $+10^{-5}$ N Na periodate; see Table I, column 4.

Fig. 7. Egg exposed for 9 minutes to hypertonic medium after pretreatment in 5×10^{-5} N Na periodate in sea water; see Table III, column 4.

Fig. 8. Egg exposed for 9 minutes to hypertonic medium after pretreatment in 5×10^{-5} N Na periodate in sea water.

Fig. 9. Underripe eggs with irregular membrane formation and no coagulation of the vitelline membrane. The expressed cortical globules have not merged with the membrane, but are visible in the perivitelline space; see Table IV, column 1.

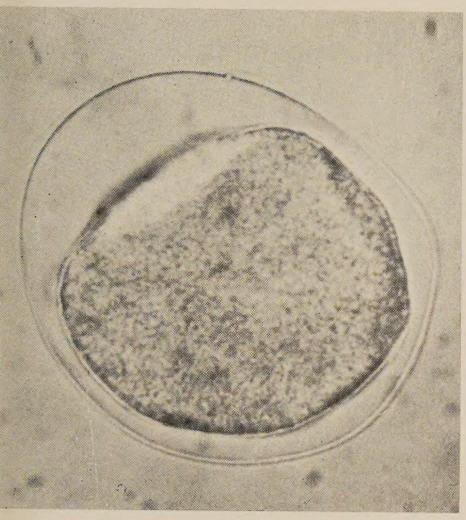

Fig. 10.

Fig. 11.

Fig. 10 Underripe egg treated with 5×10^{-5} N Na periodate. Elevation of a smooth membrane. The rather rapidly passing contraction of the egg surface still visible; see Table IV, column 2.

Fig. 11. Elongated and disintegrated oocyte after exposure to 5×10^{-5} N Na periodate.

the spermatozoa constitute the point of attack of the jelly solution, an increase in the number of the spermatozoa should tend to diminish the effect of the jelly. This has not been observed. Notwithstanding the number of spermatozoa, the membrane presents the same features in the presence of jelly solution. It is low, thin and folded, and globules are generally present between membrane and egg surface.

III. THE EFFECT OF PERIODATE IN EXPERIMENTS ON ARTIFICIAL ACTIVATION

It was shown that mere treatment of unfertilized eggs with periodate 5×10^{-5} N in sea water gave a certain low percentage of activation; see Table III ("preactivated"). It was demonstrated particularly that the treatment sensitizes the eggs to the activating effect of hypertonic sea water. The time of exposure to this medium was only 9 minutes. No changes were observed with eggs previously kept in sea water, whereas about 60 per cent activation occurred in eggs pretreated with periodate in sea water. The surface of the

TABLE II

Influence of jelly suspension and the number of spermatozoa on the mechanism preventing di- or plurispermy. The sperm suspensions were so regulated that addition of 1 drop to the sample (2 ml) gave a concentration of 2—3×10^6 spermatozoa per ml. The eggs deprived of their jelly coat were incubated for 30—40 minutes before insemination.

Survey of different samples.

2.00 ml sea water		1.75 ml sea water 0.25 » 0.043 per cent jelly solution		1.50 ml sea water 0.50 » 0.043 per cent jelly solution	
1 a	Insemination with 1 dr. sperm	2 a	Insemination with 1 dr. sperm	3 a	Insemination with 1 dr. sperm
1 b	» » 3 » »	2 b	» » 3 » »	3 b	» » 3 » »
1 c	» » 5 » »	2 c	» » 5 » »	3 c	» » 5 » »

Eggs examined 60—70 minutes after insemin. Figures give per cent of di- or plurispermic eggs and number (n) of eggs counted.

	Per cent	n		Per cent	n		Per cent	n
1 a	1±2.64	142	2 a	—	—	3 a	38±8.20	35
1 b	1±2.96	113	2 b	26±3.97	122	3 b	51±4.36	131
1 c	5±1.84	140	2 c	42±4.00	153	3 c	69±3.38	187

Differences (D) and tests of their significance, s standard deviation.

	D	s	$\frac{D}{s}$		D	s	$\frac{D}{s}$		D	s	$\frac{D}{s}$
1 c—1 a	4	±3.22	±1.24	3 c—3 a	31	±8.9	± 3.48*	2 b—1 b	25	±4.9	±5.10*
2 c—2 b	16	±5.65	±2.83	3 a—1 a	37	±8.7	± 4.25*	3 b—2 b	25	±5.9	±4.23*
3 b—3 a	13	±9.35	±1.39	3 b—1 b	50	±5.27	± 9.48*	2 c—1 c	37	±4.4	±8.40*
3 c—3 b	18	±5.55	±3.24*	3 c—1 c	64	±3.85	±16.22*	3 c—2 c	27	±5.24	±5.15*

* indicates high significance,

Table III

The action of combined treatment with periodate and hypertonic solution on unfertilized eggs. A certain number of the eggs (10 per cent) were activated already after mere exposure of the eggs to 5×10^{-5} N sodium periodate in sea water for 30—40 minutes. A low membrane was separated, the nucleus migrated to the centre of the egg and an aster appeared around the growing nucleus. These eggs are called below »preactivated». When transferred to hypertonic sea water they exhibit angular plasmolysis typical of activated eggs (9 p. 282). Eggs examined about 2 hours after transfer from hypertonic to normal sea water. The figures mean percentage, or under »n» number counted.

	Non activated	Activated			Pre-activated	n.
		With no visible membrane	With low, finely folded or granular membrane	With well elevated smooth membrane		
I. Control: Eggs 60 minutes in sea water. Thereafter 0.45 ml 2.5 N NaCl added to 2 ml egg suspension. After 9 minutes eggs transferred to normal sea water.	100	—	—	—	—	>200
II. Eggs 60 minutes in 5×10^{-5} N Na periodate in sea water (2 ml sea water + 0.1 ml 0.001 N Na periodate). Hypertonic treatment and retransfer to normal sea water as in I.	39	23	18	10 Fig. 7 and 8 (about 100 minutes after transfer from hypertonic medium).	10	216

eggs wrinkled some minutes after exposing the eggs to the hypertonic medium and after 5–6 minutes the membranes began to separate from the surface of the egg. Following the elevation of the membrane, the egg surface became smooth.

In another experiment the eggs which had been subjected to hypertonic treatment for 9 minutes were inseminated about one hour after their return to sea water. In eggs not pretreated with periodate the vitelline membrane was found to be absent in about 60 per cent of the material. This was evidenced by the fact that no membrane elevation occurred although the eggs were fertilized. An extrusion of granules and globules occurred however (7, 15). Following pretreatment with periodate a membrane was formed upon fertilization in eggs which were not previously activated as a consequence of the hypertonic treatment.

Even when the time interval between hypertonic treatment and insemina-

TABLE IV

Fertilization of eggs in the state of cytoplasmic underripeness in presence and absence of periodate. The eggs were pretreated for about 20 minutes before insemination. The form of unfertilized eggs deviated rather much from the spherical; in general they were elongated in one direction.

	1	2	3
Sea water	2.0 ml	2.0 ml	1.5 ml
Na periodate 0.001 N.....	—	0.1 »	0.5 »
Observation immediately after insemination.	In 42 per cent no visible membrane. In 68 per cent slowly and irregularly elevated membrane. Number counted: 81. Still 8–10 minutes after insemination eggs angular. Fig. 9.	In 94 per cent well elevated smooth membrane. In 6 per cent elevated but somewhat irregular membrane. Number counted: 99. Elevation of a smooth thin membrane immediately after insemin. The membrane attains normal thickness in 3–4 minutes. No granules visible. A rapidly passing contraction (Fig. 10); 2–3 min. after insemin. eggs spherical.	The eggs behaved in a way very similar to those of sample 2. A precipitation appeared which means that the concentration of periodate may have been lower than indicated.
Observations after 13–14 hours.	About 95 per cent swimming blastulæ of irregular shape, often with a deep indentation at the animal pole. About 5 per cent on the bottom, more delayed.	Swimming normal blastulæ with mesenchyme. Only about 1 per cent of irregular shape.	Blastulæ on the whole similar to those of sample 2, slightly less well developed.

tion was prolonged to 3–4 hours the same phenomena were observed. Eggs pretreated with periodate, if not activated already by the hypertonic treatment, still separated a membrane. In eggs not pretreated with periodate, fertilization was attended in 50–60 per cent of the material by extrusion of cortical material but not by regular membrane formation.

IV. EFFECT OF PERIODATE ON UNDERRIPE EGGS

The sea urchins used for the experiments reported in this paper belonged to the S-form, which is found at depths of about 30 meters. This form attains sexual maturity rather late in the summer when the litoral Z-form already approaches the end of its maturity; see (5). In the beginning of August eggs from the sea urchin of S-type may be fertilized, but the membranes

TABLE V

Fertilization of eggs in a state of cytoplasmic underripeness in presence and absence of periodate. Eggs deprived of their jelly coats. 2 drops of 1 per cent cryst. serum albumin added to each sample to avoid sticking of the eggs to the bottom of the glass dish.

	1	2
Sea water	2.00 ml	2.00 ml
Na periodate 0.001 N	—	0.15 »
Observations 50–60 min. after insemination	Monospermy: 14 per cent Di- or plurispermy: 86 per cent Number counted: 56	Monospermy: 99 per cent. Several hundred eggs examined.
Observations after 22 hours	Non segmented or arrested in early stages: 83 per cent Swimming larvae: 17 per cent The non-segmented often with nuclear divisions	Non-segmented or arrested in early stages: 13 per cent. Swimming larvae: 87 per cent.

formed are low and irregularly elevated. The further development is not normal. Pretreatment of the underripe eggs with periodate has a most striking improving effect; see Table IV. Not only is the membrane formation improved but the development proceeds in a normal way.

The underripe eggs often present a high percentage of di- or plurispermy. The control sample, 1 of the experiments recorded in Table V, gives an example of this. Again a most striking change occurred upon treatment with periodate, see column 2, Table V. The figures also demonstrate a radical improvement of the further development.

V. INFLUENCE OF PERIODATE ON THE JELLY COAT AND ITS PRECIPITATION BY CLUPEIN

Clupein precipitates the jelly coat of the sea urchin egg (9, p. 264). Treatment with periodate decreases or removes the precipitating effect of clupein; see Table VI. A certain swelling of the jelly coat occurred upon periodate treatment. It was also observed that the movements of the spermatozoa were less hampered by the jelly coat when this had been subjected to treatment with periodate.

The spermatozoa penetrate the jelly coat actively. The movements are, however, adapted to the resistance of the jelly. The tails form a narrow spiral. As a consequence of this the tails appear shortened to a varying extent. This constitutes an interesting modification of the movements of the sperma-

TABLE VI

Effect of periodate on the precipitation of the jelly coat with clupein (0.005 per cent in sea water).

	Quantity of 0.001 N sodium periodate added to 2 ml egg suspension		
	0	0.1	0.2
Effect of addition of 0.1 ml 0.1 per cent clupein to the sample (2 ml)	After 30 seconds precipitation of the jelly coat which thereafter contracts to a narrow rim. The eggs adhered strongly to the bottom of the dish. The jelly was partly converted into fine threads extending between the eggs.	Slight precipitation. No contraction ensues and the eggs do not adhere to the bottom	After 2 minutes 30 seconds slight precipitation often restricted to fractions of the jelly coat surface. Weak agglutination of the eggs.

tozoa in the free water where the tails oscillate without any directly visible shortening.

In the jelly coats pretreated with periodate the movements of the spermatozoa approach those exhibited in sea water.

VI. ACTION OF PERIODATE ON OOCYTES

From the above it follows that the eggs which have completed their maturation divisions are not injured by the exposure to periodate of the concentrations used. Conversely the oocytes are rather sensitive to this agent. After a certain time — 30–60 minutes — the oocytes elongate. The nucleus moves to one pole of the elongated cell. At the opposite pole, a deep constriction is formed. This almost pinches off a region which forms numerous extrusions of varying shape. They may appear as numerous bulges or as club or racket like appendages, Fig. 11. After 1–2 hours almost all the oocytes present in an egg suspension were transformed in the way described. These interesting phenomena could only be studied in passing.

DISCUSSION

Oxydation of jelly substance with periodate abolishes its agglutinating action on spermatozoa and its inhibitory action on blood clotting (3). The effect of heparin on blood clotting is also removed by oxidation with periodate, see (3). According to the results presented above, treatment with

periodate also removes the inhibitory action exerted by the jelly substance on the surface changes which normally occur upon activation.

Protein solutions may apparently inhibit the membrane formation in the sea urchin egg. An "osmotic" inhibition, for discussion see (11, p. 443), could possibly be involved in the effect of the jelly substance. The concentration used seems, however, too low to exert osmotic inhibition. The removal of the effect by adding periodate disproves the assumption of an osmotic inhibition exerted by the jelly substance.

The eggs of *Psammechinus miliaris* have proved to be very sensitive to the treatment with jelly substance. Not only is the coagulation and elevation of the vitelline membrane inhibited, but also the cortical material fails to undergo its normal changes; for example, the conversion of the cortical granules into rods usually does not occur. Furthermore a new effect of the jelly solution has been found, viz. its promotion of di- or plurispermy. This effect is removed simply by treatment with periodate. This may contribute to a better understanding of the mechanism which confers protection against penetration of supernumery spermatozoa. The point of attack of the periodate is most probably the carbohydrate portion of the jelly substance (2). It was demonstrated in model experiments how the viscosity of the jelly solution decreased after the addition of periodate (Fig. 1).

Several spermatozoa may enter eggs during or immediately after the maturation divisions, for references see (10, p. 14). As demonstrated above treatment with periodate removes the tendency for plurispermy in underripe eggs. Likewise the membranes become smooth and well elevated. Also in the further development the improving effect of periodate is obvious; see Table.

The data make it conceivable that an inhibitor is at work in the underripe egg. Its effect is analogous to that exerted by jelly substance on mature eggs. The question arises whether the inhibitor in the underripe egg resides in the jelly coat or inside the cytoplasmic surface. In general, underripe eggs form a defective membrane, even when deprived of their jelly coat. This suggests that an inhibitor chemically related to the jelly is present inside the cytoplasmic surface. The mild breakdown caused by periodate removes the effect of the internal inhibitor exactly as it removes the effect of the extraneous jelly solution.

As is well known, a great number of spermatozoa penetrate into inseminated oocytes. No protection against plurispermy is present in this stage.

As already pointed out the conditions for plurispermy prevail also during the maturation divisions and during a certain period after the achievement

of these divisions. The cortex of the oocytes is probably richer in the substance inhibiting the mechanism which protects against plurispermy, than is the cortex of the mature egg. This substance also inhibits the coagulation of the vitelline membrane. Even upon heavy insemination of the oocytes no coagulation of the vitelline membrane occurs. Upon insemination the oocytes exhibit the formation of numerous hyaline extrusions from the cortex (see (10) for a discussion of literature). The formation of the extrusions is probably due to local contractions of the spongioplasmic framework of the cortex. This contraction may in its turn cause the expression of more liquid components present in the meshes of the framework.

The changes of the oocytes upon periodate treatment are due to a concentration of the cortical material at one pole (the vegetal). Through the pressure exerted by the contracting cortical material, the nucleus migrates towards the pole (the animal) opposite to that where the cortical material concentrates. Sometimes the oocytes subjected to the action of periodate merely form extrusions like those appearing upon insemination. On the other hand, coagulation and constriction may occur also upon heavy insemination. These changes take place particularly in oocytes kept in a hypertonic medium (12). Activating agents thus bring about changes in oocytes which compare with those caused by periodate.

After treatment with periodate underripe eggs or eggs subjected to jelly solution get the capacity to exhibit a rapidly passing contraction upon fertilization. The inhibitory action exerted by the inherent inhibitor or the jelly solution is removed by periodate treatment. It is also noteworthy that mere pretreatment with periodate suffices to activate a certain low percentage of the ripe eggs. A considerable percentage of the eggs is, however, sensitized to the activating action of hypertonic treatment. The exposure to periodate no doubt brings the eggs into a state approaching activation, even in cases when the threshold of stimulation is not surpassed.

The slow processes which bring about cytoplasmic maturation are strongly enhanced subsequent to periodate treatment. The ripening of the egg is a process which may not be irreversible. This conclusion is supported by observations on Arbacia eggs. If ripe Arbacia eggs are stored in sea water they gradually attain a state which recalls underripeness. This seems to be due to an increase of inhibitor in the surface. The inhibition can be removed by adding serum albumin which in general improves the fertilization of underripe eggs. Underripeness probably represents a state in which the inhibitor predominates in the surface of the egg. Addition of jelly solution is an artificial method of carrying the eggs back into cytoplasmic under-

ripeness, whereas periodate so far represents the most active agent in removing the state of underripeness.

Periodate decreases strongly the inhibition of membrane coagulation. The periodate experiments suggest furthermore that the inhibitory agent is deeply engaged in the whole activation process. A number of eggs is activated upon exposure to periodate and a still greater number is sensitized to the activating effect of a hypertonic solution.

It is obvious that the data reported above are in certain respects parallel to results obtained in virus work. Hirst (2) demonstrated that the virus receptor substance of red cells was destroyed by periodate. Further significant work concerning the properties of virus receptors and inhibitors has been carried out by Burnet and his coworkers, see e.g. (1).

SUMMARY

A solution of jelly coat substance inhibits a normal differentiation of the fertilization membrane in eggs of *Psammechinus miliaris.*

The contraction of the egg surface does not occur, the coagulation of the vitelline membrane and the merging of the cortical material with this membrane are impeded. Furthermore the eggs present a varying degree of di- or plurispermy in presence of jelly coat solution.

All the effects of the jelly coat solutions which have been mentioned in the preceding paragraph are removed by addition of 5×10^{-5}N sodium periodate.

Periodate has also a certain activating effect on the ripe eggs. Particularly it sensitizes the egg to the activating effect of brief exposures to hypertonic medium.

Pretreatment of underripe eggs with periodate has a most striking improving effect on membrane formation and further development.

The jelly coats of periodate pretreated eggs are more weakly precipitated upon addition of clupein than are eggs not pretreated.

Periodate treatment has a harmful effect on the oocytes. They elongate and a deep constriction separates the oocyte into one nucleated and one non nucleated part. Numerous bulges and appendages are formed from the latter (Fig. 11).

It is inferred that underripeness is caused by the predominance of an inhibitor having a character similar to that of the jelly coat substance. The belief is expressed that the inhibitory agent is deeply engaged in the whole activation process.

This work has been supported by a grant from the Swedish Natural Science Research Council. Our thanks are due to Professor Nils Holmgren and Dr. Gunnar Gustafson, The Kristineberg Zoological Station, for generous help and supply of material, and to Dr. E. Vasseur for preparing the jelly solutions.

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ALKALINE PHOSPHATASE ACTIVITY IN DEVELOPING SEA URCHIN EGGS

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One of these writers has been engaged in investigations on the chemical changes correlated with the determination and differentiation of the sea urchin egg. Studies on the activities of certain enzymes were initiated as a further development of these investigations. Results concerning the cholin esterase activity have already been reported (1). The present work is concerned with the activity of alkaline phosphatase in different stages of development.

If sea urchin eggs in early cleavage stages are immersed in sea water with a certain content of Li, larvae develop in which the mesentoderm is excessively enlarged at the expense of the ectoderm. These effects must be due to chemical changes induced by the Li ion (7, 11). In the present investigation normal developmental stages were constantiy compared with Li-treated stages, in order to throw some light on the nature of the Li-effect.

MATERIAL AND METHODS

Fertilized eggs of *Psammechinus miliaris* were cultivated in glass trays as described by Lindahl (9). At various stages samples were taken from the homogeneous egg suspension with a calibrated glass-syringe. By using this technique exactly the same amount of eggs is removed each time from the homogeneous egg-suspension. In certain series more egg-suspension, e.g. 25 ml, was pipetted into a centrifuge tube. After centrifuging, 80 per cent of the supernatant sea water was pipetted off and discarded. Rather heavy suspensions of constant density were obtained in this way. In order to facilitate the procedure the egg-suspension was cooled before centrifuging until the larvae ceased to move. The samples, 5 or 10 ml, were immediately fixed by freezing with carbon dioxide ice in the presence of acetone and stored in an ice-box before the analysis for at most two days. The analyses were performed on total homogenates (15), obtained by grinding the thawing samples with a glass rod. It was necessary to repeat the freezing and thawing to guarantee complete homogenization. Immediately after homogenizing aliquots of the homogenates were

analyzed, whereby sodium phenylphosphate in a veronal buffer of pH 9.65 was used as substrate (2). As the sea water of the homogenates contains Mg no extra addition of this ion for the activation of the enzyme was necessary. The time of incubation was 30 or 60 minutes, and the temperature of incubation varied between 18° and 25°. In each series all samples were treated in the same way.

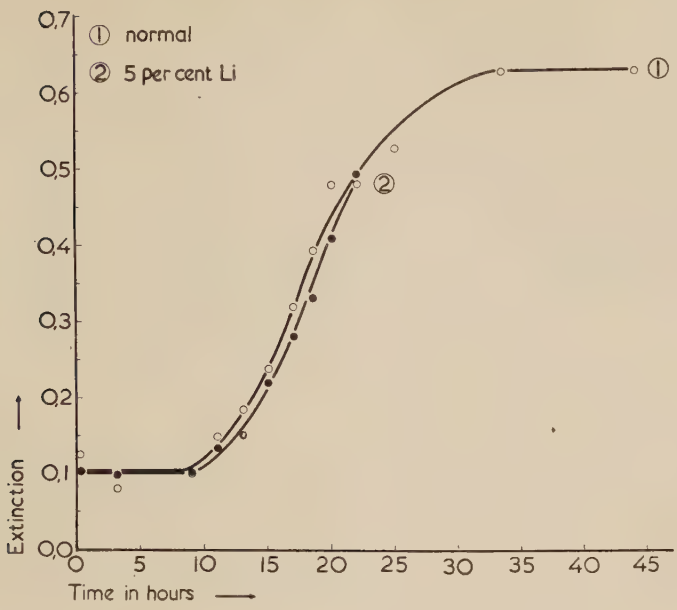
RESULTS

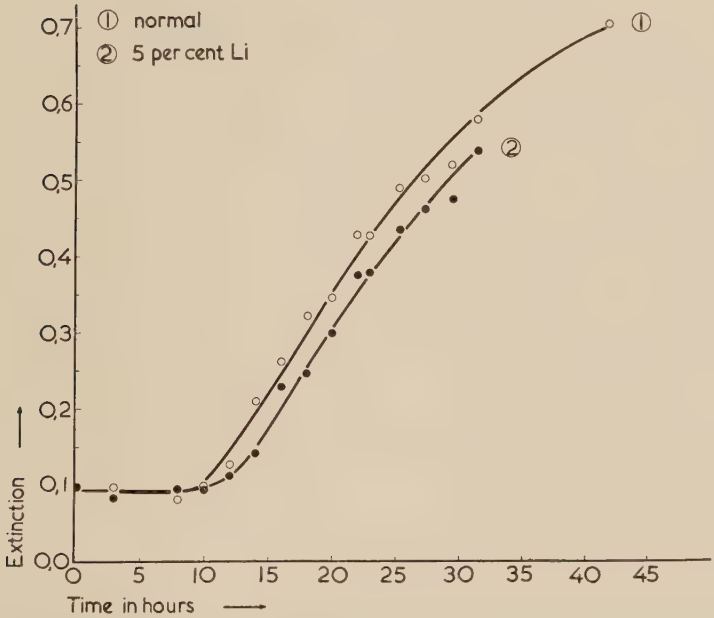
The main results of different series are identical. During the first seven or nine hours, i.e. in the cleavage stages, the activity of alkaline phosphatase is rather constant and comparatively weak. In certain experiments, however, a weak continuous rise was recorded. A rapid exponential rise of the activity begins after the appearance of the primary mesenchyme cells in the blastocoele. This rise gradually becomes less steep and ceases after thirty hours in one of the curves reproduced (Fig. 1), whereas in the other curve (Fig. 2), it still continues after fortytwo hours. In this batch where the rise in phosphatase activity ceased early, the arms of the plutei were poorly developed. Conversely, plutei with well developed arms were present in the batch where the rise in phosphatase activity continued after fortytwo hours. It is thus obvious that the differences between the curves in Figs. 1 and 2 are correlated with morphological differences between the two batches of eggs.

Li-treatment (5 per cent isotonic Li-solution, 95 per cent sea water of 2.3 per cent salinity) gave rise to strongly vegetalized larvae, but did not change the picture essentially. The Li-curve was always parallel to the normal curve, but the rise occurred somewhat later. The Li-series were, however, interrupted earlier than the normal series, as the Li-larvae show signs of beginning disorder in later stages. In one Li-experiment (Fig. 2), the activity continued to rise until the experiment was interrupted after thirtyone hours, although the larvae appeared during the last five hours increasingly compact and dark. Experiments with stronger Li-treatment during the cleavage stages followed by further cultivation in normal sea water have not as yet been carried out. Egg homogenates incubated with Li for 30 minutes before the addition of substrate had the same alkaline phosphatase activity as had the control without Li.

DISCUSSION

The alkaline phosphatase activity is especially high in cells engaged in an intensive protein synthesis (3, 4). In embryonic tissues the activity is weak during earlier stages of development but becomes intense at the beginning





Figs. 1 and 2. Activity of alcaline phosphatase at different times after fertilization expressed by the extinction measured on the digests after treatment with the Folin phenol reagent. Circles control, dots Li-treated larvae.

of differentiation. This seems to be in agreement with the results on *Psammechinus* with phenylphosphate as substrate and with the results reported by Mazia *et al.* (14) on *Arbacia* with glycerophosphate as substrate. It also corresponds with the histochemical results of Krugelis (13) on *Arbacia*.

The low activity of the alkaline phosphatase during the cleavage stages, when the extent of the ectoderm and entoderm is determined (8, 9), coincides with a very low activity of the amino acid metabolism (6). No changes occur in the antigen composition of the egg (16). It thus seems that the protein metabolism is quantitatively unimportant during the period of determination. Perhaps very scarse populations of protein molecules with a new specificity are built up during the determination period under the influence of the animal and vegetal gradients of action. When at the beginning of gastrulation the conditions for a rapid protein synthesis are realized, e.g. by an increased activity of the alkaline phosphatase, a rapid growth of these populations might take place (17). This is reflected in changes of the chemical, serological and morphogenetical properties of the larvae. The total amount of various amino acids changes and the free glutamine disappears (6). A new antigen arises (16) and the determination of the dorso ventral organization of the larvae becomes irrevocable (5).

It is evident that, following Li-treatment, the formation of some fibrous structure proteins has been checked, as no ciliar tuft develops and as the cell stretching is incomplete. Li does not interfere with the formation of phosphatase or with its activity, but it may interfere with an earlier link in the chain of processes which lead to the elaboration of the fibrous structure proteins in question.

According to the histochemical investigation of Krugelis on *Arbacia* the rise in phosphatase activity seems to be localized in the nuclei. The histochemical results must, however, be very cautiously interpreted (18). Thus the role of the cytoplasm in the total phosphatase activity of the eggs does not seem to be definitely determined. The method of Krugelis (12) in which desoyribo- and ribo-nucleic acids depolymerized by nuclease are used as substrate for phosphodiesterases in the nucleus and cytoplasm may prove to be of value in further quantitative studies.

SUMMARY

1. The alkaline phosphatase activity in the sea urchin egg is very weak until the beginning of gastrulation, whereafter an exponential rise appears.

2. Li has no effect on the result besides a slight delay of the beginning of the rise.

3. The amino acid and protein metabolism etc. of the sea urchin egg are discussed in relation to the results presented above.

The authors wish to express their most cordial thanks to Professor John Runnström for the great interest he has shown in our work. We also owe a great debt of gratitude to Professor Nils Holmgren and Doctor Gunnar Gustafson, the Kristineberg Zoological Station, for their never failing generosity.

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INCORPORATION IN VIVO OF ^{15}N-LABELED GLYCINE INTO LIVER FRACTIONS OF NEWLY HATCHED CHICKS

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As is already well known, it is possible to isolate, by means of differential centrifugation, the following cell fractions from tissue homogenates (5, 10): (1) nuclei, (2) large granules containing mostly mitochondria, (3) microsomes, and (4) cell fluid. These fractions are fairly well distinct from each other from morphological, analytical, and functional points of view, although overlapping intermediates are found (3, 11).

The mitochondria are the bearers of several important enzyme systems (4, 10, 13, 19, 22, 23, 24). In the present connection it is of special interest to notice that the production and transmission of phosphate-bound energy is centered in this fraction (3, 8, 21). The submicroscopic microsomes, on the other hand, like viruses, have a very incomplete enzyme equipment (9, 18), but contain a large part of the ribonucleic acid of the cell (5, 10).

Both of these two granular cytoplasmic fractions are very labile. They diminish rapidly in fasting animals and reappear when a protein-rich diet is given (14, 15). It is believed that the plasmagenes are carried by the microsomes (15). In rapidly cleaving embryonic cells with high contents of ribonucleic acid a rapid reduplication of the microsomes can therefore be expected.

Most likely, the syntheses of all structural elements of the cell are closely interdependent. If isotope containing synthesis products from one cell fraction are readily utilized by another fraction, an equilibrium between the fractions in the rate of isotope incorporation will rapidly be obtained in isotope experiments. In the present investigation, where the incorporation of ^{15}N-labeled glycine into the liver fractions of newly hatched chicks was studied, a prevailing interest was therefore attached to the first short period after the administration of the isotope.

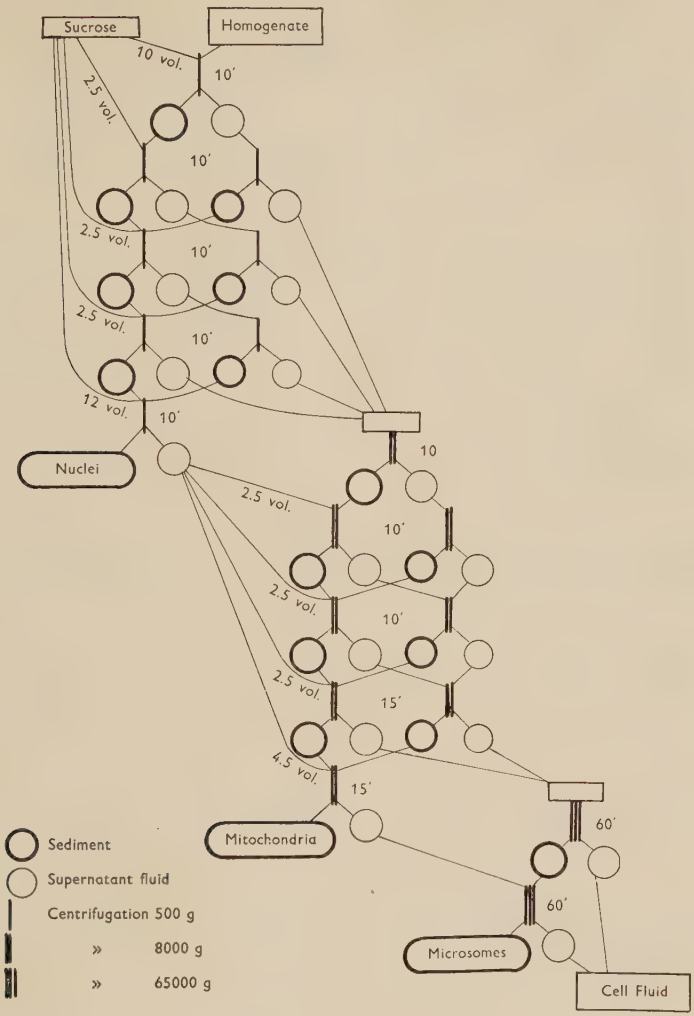

Fig. 1. Diagrammatic scheme of the chick liver fractionations. Centrifugal forces are calculated at the middle of the tubes.

EXPERIMENTAL

Glycine was synthesized according to Schoenheimer & Ratner (25) from ammonia containing 61 atom percent excess ^{15}N. Each chick ("White Leghorn", weight about 38 g) received an injection into one wing vein of 26.8 mg glycine (5 mg N) and was then kept at constant temperature, 38° C. The chicks were killed by decapitation

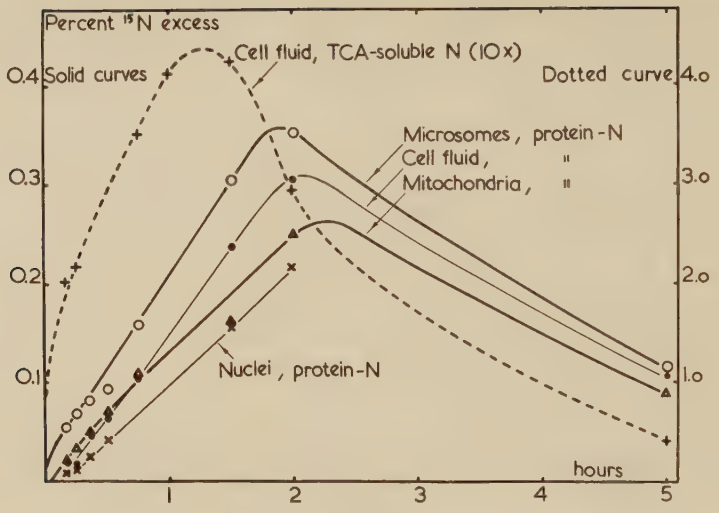

Fig. 2. Isotope concentrations in different chick liver fractions found at various times after the intravenous administration of ^{15}N-labeled glycine (5 mg N, 61 percent ^{15}N excess).

at different times (10 minutes to 5 hours) after the administration of the isotope. The livers (weights about 900 mg) were immediately removed, dissected, and thoroughly washed in ice-cold Chambers-solution (5). They were homogenized, strained, and fractionated in isotonic sucrose-solution, pH 8.5 (Fig. 1). The whole procedure was carried out at 0–3° C.

The fractions were precipitated with ice-cold 10 percent trichloroacetic acid (TCA), centrifuged, and thoroughly washed once in ice-cold 2.5 percent TCA containing 0.1 N *ordinary glycine*, and then twice in pure ice-cold 2.5 per cent TCA. Lipids and nucleic acids were removed according to the methods of Schneider (20), and the nitrogen of the residue, which was considered as protein-nitrogen, was determined and analyzed for excess ^{15}N.[1] All washings and extractions were made by grinding the sediments with an air-driven, rotating glass-rod.

The nucleic acid contents of these same fractions were determined according to the methods of Schneider (20) and Euler & Hahn (6).

RESULTS

The isotope experiments are illustrated by Fig. 2. The labeled glycine was rapidly transferred to the liver, where it appeared in high concentration

[1] By the courtesy of Professor E. Hammarsten, the isotope determinations were performed at his laboratory.

in a TCA-soluble form. The isotope concentration of the total TCA-soluble nitrogen reached a maximum value after about one hour.

The isotope was readily incorporated into the liver proteins. The isotope incorporation into the microsome proteins started more rapidly than the incorporation into the proteins of the other cell fractions.

Cytoplasm is considered to be more or less closely intermingled with a fine network of submicroscopic fibrils (7). It has been shown that these cytoplasmic fibrils are cross-striated and contain submicroscopic granules alternating with birefringent polypeptide chains (17), possibly built out from the granules (16). Especially in rapidly growing cells the submicroscopic granules are rich in ribonucleic acid (16). Most likely they constitute an essential part of the microsome fraction obtained by differential centrifugation. The rapid initial rate of the isotope incorporation into the microsome fraction is in close agreement with the idea that the submicroscopic granules, isolated from disintegrated cytoplasmic fibrils, include a charge of rapidly synthesized and continuously emitted proteins, e.g. the proximal fragments of the polypeptide chains mentioned above.

The high rate of isotope uptake by the microsome proteins is in agreement also with the higher ribonucleic acid content of the microsome fraction (Table I) (2). It is of special interest that the present experiments indicate that the ribonucleic acid is related not only to the transformation of polypeptide chains into proteins of specific structure, but also to the initial incorporation of amino acids into peptide chains (26). The immediate availability of ribonucleic acid is evidently more important for this peptide synthesis than a close proximity to energy-producing enzyme systems.

TABLE I

Distribution of TCA-soluble N, nucleic acids, and final protein-N in the liver fractions

Cell fraction	TCA-soluble N, mg/liver	Nucleic acids, mg/liver	Protein-N, mg/liver
Nuclei	(0.07)	1.2	1.4
Mitochondria	(0.04)	0.56	1.7
Microsomes	(0.04)	1.8	1.7
Cell fluid	1.2	0.71	4.5

The later average isotope uptake of the microsome proteins was more rapid than that of the other granular cell fractions. After an initial delay, the isotope concentration of the cell fluid proteins increased at a rate almost parallel to that of the microsome proteins. Thereby they surpassed the mitochondria proteins in spite of their considerably higher nitrogen content (Table I). The

nuclear fraction (containing only small impurities of tissue fragments) had the smallest average isotope uptake rate.

No efforts were made to determine in detail the turning-points of the isotope incorporation into the protein fractions. In the related experiments there was a culmination in the cytoplasmic protein fractions about two hours after the administration of the isotope. The cell fluid and the mitochondria reached their maximum values slightly later than the microsome fraction.

The average isotope uptake rate is not necessarily a very adequate measure of the mean velocity of the protein synthesis. A divergent amino acid composition of a cell fraction may influence the isotope uptake. Moreover, the velocity of protein synthesis is probably not quite the same throughout a cell fraction or any particular granule. However, preliminary solubility experiments, performed to differentiate the proteins of the cell fractions, gave protein samples of the same isotope concentrations.

Both the initial form of the microsome curve, and the fact that, after an initial delay, the cell fluid curve became parallel to it, make it very probable that the microsomes contribute in a high degree to the surprisingly high isotope level of the cell fluid proteins. It was thought that the microsomes contribute also to the proteins of the mitochondria. During the course of the present work it has been learned, however, that even isolated mitochondria can perform a synthesis of proteins *in vitro* (1).

SUMMARY

In the livers of newly hatched chicks, ^{15}N from labeled glycine is incorporated *in vivo* into the microsome proteins earlier and at a more rapid rate than into the proteins of any other cell fraction isolated by differential centrifugation. The microsomes, moreover, probably contribute in a high degree to the surprisingly high isotope level of the considerable amounts of cell fluid proteins.

Since the microsomes are especially rich in ribonucleic acid, and the production of phosphate-bound energy is centered in the mitochondria, the experiments indicate that the immediate availability of ribonucleic acid is more important for the formation of proteins than a close proximity to energy-producing enzyme systems.

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STUDIES ON MITOCHONDRIA: I. THE ASSOCIATION OF CYCLOPHORASE WITH MITOCHONDRIA[1]

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The integration of the chemical events concerned with the oxidation of pyruvic acid into the citric acid cycle (14, 15) indicates that the underlying enzymes have a special arrangement with respect to one another. The very nature of the cycle implies a degree of organization in which the constituent enzymes are held in a precise structural relationship. Recently a system has been obtained from animal tissues, which catalyzes the complete oxidation of pyruvic acid, fatty acids and certain amino acids to carbon dioxide and water by way of the citric acid cycle (6, 25, 5). Since the prime function of this system is to implement the citric acid cycle, it has been called the "cyclophorase system" by Green, Loomis and Auerbach (6). The extensive investigations of these workers have led them to the view that the enzymes of the cyclophorase system are not a random collection of discrete enzymes, but are exactly fitted together as in a mosaic. The unit is no longer the individual enzyme, but an hierarchy of enzymes, mutually adapted kinetically and spatially. This complex unit comprises the cyclophorase system.

The earlier studies on cyclophorase established that it existed among the cellular particulates, which contributed to its integrity, although a definite cellular structure was not identified. Other workers (23, 10, 24, 13) have demonstrated that oxidative systems have a restricted localization in the subcellular granules obtained from tissue homogenates by cell fractionation. They demonstrated that the citric acid oxidative cycle is associated with the mitochondria. The purpose of the present communication is to present evidence establishing that mitochondria are the structural elements which sustain oxidative and ancillary enzymes lying within the province of the cyclophorase system.

[1] This investigation was supported in part by a grant from the Commonwealth Fund.

EXPERIMENTAL METHODS

The specimens of cyclophorase suspensions employed were prepared in the conventional manner (6) under conditions optimal for oxidative activity, and were routinely used as the thrice washed residue, designated R_3. Preparations were obtained from rabbit liver, kidney, heart and skeletal muscles. Except during the final microscopic study, the suspensions were regularly maintained at 0° C, unless a manipulation specifically required the use of higher temperatures. These suspensions were usually made in 0.9 per cent potassium chloride. In several instances, however, they were made in either 17 per cent or 30 per cent sucrose to facilitate the separation of the cytological units by differential centrifugation.

Several fractionations of cyclophorase suspensions were made at 0° C in 17 per cent sucrose. An initial centrifugation at 200 × g for 15 minutes[1] removed the bulk of the chromatin threads, which were set aside. The supernate from this centrifugation were subjected to 4,500 × g for 30 minutes, which deposited the bulk of the mitochondria, with a few residual chromosomes. This sediment was resuspended and after a preliminary centrifugation at 200 × g to remove the remaining chromosomes, was given a final wash and resuspended in a small volume of 30 per cent sucrose. The "microsomes", which remained suspended in the first supernate (S_1) were freed of any contaminating mitochondria by a centrifugation at 25,000 × g for 30 minutes, and were concentrated by centrifuging at 25,000 × g for 60–90 minutes.

Various transforming agents were applied to the cyclophorase preparations by the procedures of Huennekens and Green (11). After a suitable period of exposure, the suspensions were studied cytologically for morphological alterations. In certain instances the oxidative activity was concurrently tested manometrically by Warburg technique, and in others specific enzymes such as succinoxidase, were investigated by the Thunberg procedure.

Experiments were designed to determine the cyclophorase activity as a function of the number of mitochondria. Aliquots of the suspension were tested for rate of oxidation in presence of α-ketoglutarate (6). Other aliquots were suitably diluted to 250–500 times with 0.9 per cent potassium chloride for mitochondrial counts. Samples were measured by Breed and Brew pipettes onto a marked 18 × 18 mm area on a glass slide, and evenly spread over this area. The wet slides were exposed to fumes of 4 per cent osmium tetroxide at room temperature. After 6–12 hours, they were stained selectively by Fast Green FCF (8). The mitochondria were counted with a Whipple disc, the dimensions of which were determined by a stage micrometer scale. For each specimen at least three slides were prepared and in each slide 30 fields were counted.[2] Since the volume of suspension, the area occupied, and the number of

[1] In the various centrifugations an International Refrigerated Centrifuge PR–1 was used. For the low gravity manipulations, an angle head of 20 centimeters diameter was employed, and for the higher gravity experiments, the Multispeed attachment, number 295, replaced the angle head. The Centrifugal forces designated indicate those at the distal extremities of the tubes employed.

[2] For light microscopy the Spencer Microscope R-4-L was used throughout, and all photographs were taken with use of a Bausch and Lomb Hyperplane (10 ×) ocular. Phase microscopy was carried out with a Spencer Phase system which included a turret condenser, and 1.8 mm lens for Dark-M, Light-M, and B-Minus Contrasts.

mitochondria per area were known, it was possible to compute the number of mitochondria in a unit volume of original undiluted suspension.

The cytological studies were made by phase microscopy on fresh suspensions and by light microscopy on fixed smears. The B-Minus contrast had sharpest definition and aided study of contour in the sucrose solutions, whereas the Dark M demonstrated swollen structures most suitably. For routine examinations the smears were fixed and stained by the Papanicolaou (19) technique. This facilitated examination for whole cells and nuclear structures. The smears were also stained by the Feulgen method to identify chromatin fragments. Other smears, fixed by the fumes of 4 per cent osmium tetroxide were selectively stained for mitochondria. The mitochondrial stains included those of Altman (1), Cowdry (3), Benda (2), and Volkonsky (26), which were later replaced by another procedure using aniline Fast Green FCF (8). In several instances whole suspensions of renal cyclophorase were centrifuged to form tight pellets, which were fixed in 10 per cent formalin containing an M/10 concentration of lanthanum acetate. The pellet was imbedded in paraffin, sectioned and stained by Mallory's azure-methylene blue and phloxine method (18). Other sections were stained by the periodic acid procedure of McManus (17) to delineate basement membranes seen in kidney preparations. The Mallory stain helped to discern cell contours, whereas the periodic acid technique demonstrated an occasional intact brush border indicating an unruptured cell.

RESULTS

I. *Cytological examination of cyclophorase preparations*

Many oxidatively active samples of cyclophorase (renal, hepatic, and cardiac) were examined for intact cells. The techniques employed were such as had been elaborated to facilitate the study of whole cells. By routine use of these procedures on many preparations, the presence of parenchymal cells was regularly excluded. These observations were strengthened by the frequent examination of cyclophorase preparations which had been suitably fixed, imbedded in paraffin and after sectioning, were suitably stained. In addition numerous suspensions were inspected for cells by phase microscopy. This latter type of examination was made routinely whenever correlation was made between enzymatic activity and the concentrations of different subcellular granules in the active suspensions. As a result of these studies it was settled securely that the catalytic activity of cyclophorase as prepared under standard conditions, is independent of whole cells and therefore, referable only to the subcellular residues. In cyclophorase obtained from kidney intact cells were not observed, although the convoluted tubules were converted by homogenization into short, recognizable segments. Such segments (Fig. 1) consisted of sleeves of basement membrane, which

ensheathed and retained aggregates of subcellular granules derived from the completely shattered tubular cells.

II. *Lack of correlation of cyclophorase with nuclei*

The fragility of cyclophorase preparations as indicated by the necessity of working with fresh tissues rapidly in the cold, suggested an association with some delicate subcellular unit. Frequent inspections of preparations of cyclophorase revealed that all the nuclei were converted into elongated fragments of the variety which Ris and Mirsky have identified as intermitotic chromosomes (20). This observation excluded any contribution to the cyclophorase activity by whole nuclei, but did not eliminate the participation of the chromosomes. When chromosomes were concentrated and separated from the remaining subcellular granules by centrifugation and washing in low gravitational fields, manometric tests disclosed essentially no oxidative activity. Conversely the removal of the chromosomes from the residues, so thoroughly that they were not detectable by the Feulgen reaction (Table I), eliminated a portion but not the whole of the original oxidative activity. The portion removed by fractional centrifugation included, especially in the case of the kidney system, elements other than chromatin threads. Thus, the oxidative activity of cyclophorase was manifest in preparations free of chromatin threads. However, a loss of the original activity attends the elimination of the chromosomes. This loss may be attributed to the removal of active elements as well as chromatin threads, or to the fact that the chromosomes make a secondary contribution to cyclophorase activity. Schneider and Potter (13) reported that their nuclear fractions manifest only a slight oxidative activity, and hold the view that these fractions are still contaminated with other cell particulates.

In further experiments it was found that prolongation of homogenization up to 5 minutes or more inactivated cyclophorase, although such treatment had no visible effect on the structure of the chromosomes. This strengthened the view that cyclophorase activity was independent of either nuclei or chromosomes.

III. *Correlation of cyclophorase with mitochondria*

It was noticed that the first supernate (S_1) obtained in the preparation of cyclophorase often showed a slight oxidative activity toward components of the citric acid cycle. This fraction was found to be composed mainly

TABLE I

Oxygen uptake of cyclophorase prepared by differential centrifugation in sucrose medium

Sample	Original homogenate	Non-chromosomal fraction	Oxygen consumption of non-chromosomal fraction as per cent of original homogenate
	μ atoms oxygen	μ atoms oxygen	
1	49.0	28.0	57.0
2	43.0	24.0	58.0
3	36.0	22.0	61.0
4	28.0	6.0	21.4
5	32.0	18.6	58.1
6	49.0	10.1	20.6

The suspensions were prepared in 17 per cent sucrose, and cyclophorase was freed of chromosomes by low speed centrifugation. The preparations were tested for oxidative activity in Warburg flasks which contained 1.5 ml of cyclophorase from liver at the third residue stage (R_3L), 0.3 ml. of 0.1 M phosphate buffer of pH 7.3, 0.3 ml of 0.01 M adenosine-5-phosphate, 0.2 ml of 0.02 M magnesium chloride and water to a final volume of 3 ml. The experimental vessels contained 0.2 ml of 0.1 M α-ketoglutarate. The center well contained alkali. The gas space was filled with oxygen and the vessels equilibrated in a water bath at 38° C. Oxygen uptake for the first 5 minutes was presumed to equal that for the subsequent 5 minutes. The oxygen consumption was determined over a period of 30 minutes.

of "microsomes" with few discernible mitochondria. Centrifugation at 25,000 ×g for 15 minutes in the high speed head removed the cyclophorase oxidative activity from the suspension. Such treatment left the bulk of the microsomal elements in the supernatant, but removed all of the mitochondria.

The simultaneous elimination of mitochondria and cyclophorase activity indicated an interdependence. On the other hand, the absence of cyclophorase activity in the suspension composed of "microsomes" alone, precluded the essential participation of the microsomal particles in the cyclophorase system. This negative test for cyclophorase activity does not prove the absence of the enzymes which are responsible for cyclophorase activity. On the contrary the "microsomal" suspension contains qualitatively all the enzymatic activities of the original cyclophorase. There is lacking, however, the ability to oxidize completely any member of the citric acid cycle to carbon dioxide and water, under well defined conditions. The one step oxidations are still exhibited by the "microsomal" fraction when supplemented with cofactors, whereas the catalytic tests reveal absence of the serial oxidative activity characteristic of the cyclophorase system.

Mitochondria were observed in all cyclophorase preparations. They were

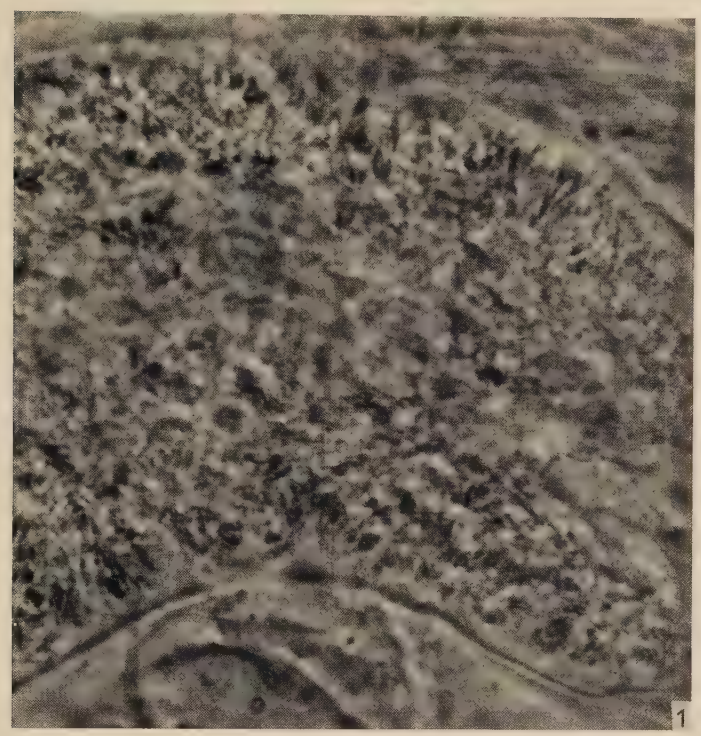

Fig. 1. A fragment of a renal convoluted tubule suspended in 17 per cent sucrose is shown. The masses of rod-shaped mitochondria, irregularly arranged and filling the tubule are not confined by cell borders or mixed with nuclei. The highly refractile basement membrane forms a limiting structure to the cell-free mitochondria, and, since the cell membranes are ruptured, constitutes the only barrier between the mitochondria and the medium. By variation of depth of focus the membrane is seen to encircle the mitochondrial mass like a cylinder.
Phase Contrast, B-Minus. 1,400 × Magnification.

numerically the predominant subcellular units in kidney and liver cyclophorase, whereas in heart they were the only recognizable pre-existant structural unit. With the Fast Green staining procedure (8), the mitochondria were counted. It was also possible by phase microscopy and the use of this stain to detect alterations of the mitochondria under the influence of different agents. The cyclophorase activity was therefore correlated with the number and state of the mitochondria after staggered intervals of homogenization ranging within the limits of 1–10 minutes. Liver cyclophorase was used throughout these experiments because the retention of mitochondria within the tubular fragments in kidney prevented accurate counting. Table II

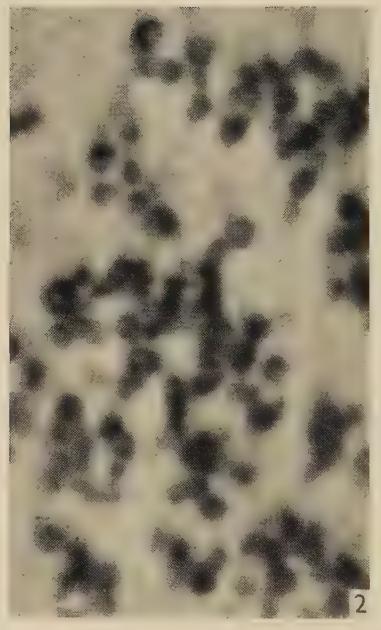

Fig. 2. Cyclophorase was prepared in 0.9 per cent KCl, with 30 seconds homogenization. Samples were smeared on glass slides, fixed by vapors of 4 per cent osmium tetroxide and stained with Aniline Fast Green FCF. The mitochondria acquired the green dye selectively. Its concentration in the mitochondria is uneven and divides the units into two areas: one area is a densely staining crescent, the other a lighter staining portion of the sphere. 1,500 × Magnification. 3 × Enlarged.

Fig. 3. Cyclophorase prepared in 0.9 per cent KCl, with 3 minutes homogenization, is fixed and stained in the same manner and at the same time as the preparation seen in Fig. 2. A few mitochondria have the composite structure seen in the preceding 30 second homogenate. They are mostly larger, lack internal concentration of dye and manifest a poorer differential affinity for the Green dye. This preparation has lost about half its enzymatic or cyclophorase activity.
1,500 × Magnification. 3 × Enlarged.

illustrates that the catalytic activity, the numbers and the state of mitochondria were varied by the different times of homogenization. Cyclophorase activity is reduced practically to zero after blendorizing for 5 minutes and the mitochondrial count is at most halved. At first glance there appears to be no correlation between cyclophorase activity and the number of mitochondria. However, examination of the various preparations which had been blendorized for successive periods of 1, 3, and 5 minutes disclosed significant changes in the 3 and 5 minute homogenates as contrasted with the 1 minute control. In the former suspensions the majority of mitochondria had lost their characteristic composite structure (Fig. 2 and 3), and assumed a homo-

TABLE II

Correlation of oxygen uptake and mitochondrial content of cyclophorase

Time of homogenization	Mitochondrial count/ml.	Alteration of structure[1]	Oxygen uptake μ atoms per 30 min.
	Experiment 1		
1 minutes	58×10^{10}	Slight	42.4
3 »	56×10^{10}	Marked	21.8
5 »	29×10^{10}	Extensive	0
	Experiment 2		
1 minutes	50×10^{10}	Slight	27.4
5 »	27.6×10^{10}	Extensive	0

The conditions of oxygen uptake estimation are those recorded in Table I. Liver cyclophorase was used as the third washed residue (R_3L). The mitochondria were counted by the procedure described under experimental methods.

geneous undifferentiated appearance; simultaneously many swelled to a diameter nearly twice that seen in the control suspension. The association of this phenomenon with a decrement of oxidative activity was regular and the extent of alteration paralleled closely the drop in catalytic activity. Thus the state of the mitochondria manifested a more precise relation to the cyclophorase activity than did the actual number of units. This was more extensively tested by the use of transforming agents (11).

Huennekens and Green (11) describe a series of chemical agents and physical conditions which transform the conjugated pyridinoprotein enzymes of the cyclophorase complex into dissociated, classical form. Cyclophorase suspensions were submitted to the action of several such agents and the effects upon the mitochondria determined. Most transforming agents and procedures produced no change in either chromosome or "microsomes", whereas all altered the mitochondria to some extent. Capryl alcohol and high concentrations of ammonium sulfate caused crenation and shriveling of mitochondria; on the other hand deionized water, high concentrations of urea (30 per cent), and exposure to 38° C. induced an irreversible swelling. In the presence of gramicidin and dinitrophenol there was little conspicuous alteration in the contour of the mitochondria. These agents obliterated the internal structure of the units. Sodium cholate altered mitochondria more dramatically than any other agent tested. In its presence the mitochondria swelled voluminously,

[1] For details of structural alteration see the description in the text.

TABLE III

P^{32} incorporation in mitochondria and "microsomes"

Substrate	Radio-activity counts/minute		
	Mitochondria 0°	Mitochondria 38°	Microsomes 0°
Blank	118	101	52
α-Ketoglutarate	854	768	56
Succinate	844	758	52
Malate	794	708	51

The suspensions of mitochondria and "microsomes" were prepared in 17 per cent sucrose and separated by the procedure of differential centrifugation described in the section on experimental methods. Absence of contaminating chromosomes was confirmed by the negative Feulgen reaction. All suspensions were tested manometrically at 38° for oxidative activity under conditions described in Table I, except that both succinate and malate replaced the α-ketoglutarate as substrate in the appropriate experiments. At the end of 15 minutes incubation at 38° C. (and of 30 minutes at 0° C.) the flasks were chilled and the contents washed twice with 50 ml. of 0.9 per cent potassium chloride at 0° C. The washed gel was dissolved in 5 ml. of 8 per cent potassium hydroxide and the radioactivity in a 1 ml aliquot determined. For further details of experiments with P^{32} incorporation by cyclophorase suspensions see the paper by Green, D. E., Atchley, W. A., Nordmann, J., and Teply, L. J., *Arch. Biochem.*, **24**, No. 2, 359 (1949).

with the formation of bizarre internal configurations. All the transforming agents, in the range of concentrations tested, uniformly caused both loss of cyclophorase activity and structural changes of the mitochondria. Slow freezing and thawing is of particular interest because it completely obliterated all structure and cyclophorase activity. Yet all the individual oxidases survive the treatments, in so far as they exhibited activity in the presence of appropriate cofactors. Uniformly the alteration of mitochondria and cessation of cyclophorase activity were simultaneous. Loss of cyclophorase activity could not be correlated with corresponding change in any other subcellular unit in the preparations.

A correlation was made not only between cyclophorase activity and mitochondria, but also between oxidative phosphorylation and the presence of these units. In Table III, data are presented to show that the incorporation of inorganic P^{32} during oxidation (a diagnostic of an intact cyclophorase system) is characteristic of the mitochondria but not of the "microsomes". Both oxidative phosphorylation and cyclophorase activity are phenomena which are found only in fractions containing mitochondria. It is significant that all transforming agents tested have the property of blocking this process of oxidative phosphorylation just as they abolish oxidation.

TABLE IV

Distribution of succinoxidase activity[1] as a function of the time of homogenization

Experiment	Duration of homogenization	Activity of residue	Activity of supernate	Activity in residue / Activity in supernate
		Units succinoxidase	Units succinoxidase	
A	30 seconds	1,420	1,140	1.25
B	1 minutes	795	2,080	0.38
C	2 »	974	1,740	0.56
D	7 »	136	1,880	0.072

The test for oxidase activity was carried out in evacuated Thunberg tubes containing 0.5 ml. of liver residue or 1 ml. of supernate, 0.1 ml. of M succinate; 0.3 ml. M/10 phosphate buffer of pH 7.5 and 0.5 ml. of 0.1 per cent 2,6-dichlorophenolidophenol. The suspension was incubated at 38° C. for 2 minutes before mixings. Each experiment had as a blank a tube containing all ingredients except succinate.

In view of the fact that "microsomes" contain the oxidases present in cyclophorase (and yet cannot catalyze the complete oxidation of citric acid intermediates to carbon dioxide and water) studies were made of the relationship of the mitochondria to "microsomes". Succinoxidase was selected, because it is generally agreed to reside in the mitochondria, (22, 9, 21). The proportion of this enzyme as distributed between supernate and residue was determined in preparations exposed to varying durations of homogenization (Table IV). The experiments demonstrated that after 10 minutes of homogenization essentially all the succinoxidase activity was in the supernate (microsome fraction), whereas after 30 seconds homogenization more than half the activity remained in the residue (cyclophorase fraction). Briefer homogenizations were impractical because many cells were not ruptured. There is apparently a progressive shift of the enzyme from the residue into the supernate, paralleled by a decline in the mitochondria and quantity of residue, with corresponding increase in the "microsomes" and the protein (dry weight) of the supernate. It was observed that about 15 per cent of the original enzyme activity is lost due to prolonged homogenization, although such a decrement does not obscure the trend. Several other oxidases tested behaved similarly, but differed in the ease or difficulty with which they were detached from the cyclophorase system (12). It appeared that the shearing action of the blendor reduced the size of the particles of the mitochondrial-cyclophorase residue into fine, disrupted fragments of the dimensions of the

[1] The unit of succinoxidase activity is defined as the amount of enzyme which will reduce 0.5 ml. of 0.1 per cent 2,6-dichlorophenolindophenol in 1,000 seconds at 38° C.

"microsomes". From such experiments it could not be deduced that all "microsomes" were derived from the mitochondria, since with the briefest practical homogenization, "microsomes" were found in the first supernate and were associated with 45 per cent of the total succinoxidase activity. A proportion of the "microsomes" in the first supernate could indeed be referred to the comminution brought about by the initial 30 second homogenization, but there is no feasibility of extrapolating back to conditions in the intact cell. However, it is clear that transition from mitochondria into "microsomes" is accompanied by loss of cyclophorase activity without significant equivalent destruction of individual enzymatic activity.

DISCUSSION

The site of cyclophorase activity in mitochondria has been established by the unique dependence of one upon the integrity of the other, and by a close correlation between the distribution of mitochondria and cyclophorase in fractions from tissue homogenates. In view of evidence for internal structure in the mitochondria (7), and of the fact that dispersion of this structure accompanies inactivation of cyclophorase, it is suggested that integrity of mitochondrial structure and of cyclophorase activity represent two aspects of the same phenomenon.

The question arises whether mitochondria are uniform in all tissue cells and even the same cell. They may be replicas of one fundamental unit, or may vary according to the tissue of origin. Available evidence concerning structure and function is statistical in nature and permits no answer to this problem. By our experiments and observations all mitochondria appear and behave identically. Because the density of the suspension is great (billions per ml) a decision concerning subtypes is clearly precluded. The investigations of Green and his colleagues indicate that mitochondria from all tissues share a fundamental enzyme pattern, but other studies (16, 4) suggest that certain enzymes are found only in mitochondria of particular tissues.

SUMMARY

The study of the activity and cytology of the cyclophorase system under a variety of conditions has established that mitochondria are the morphologic units of the cyclophorase system. A close correlation was also observed between the state and number of mitochondria and the extent of cyclophorase activity.

ACKNOWLEDGEMENT

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STUDIES ON MITOCHONDRIA: II. THE STRUCTURE OF MITOCHONDRIA IN RELATION TO ENZYMATIC ACTIVITY[1]

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INTRODUCTION

The significance of the mitochondria in the phenomena of cellular respiration has been accentuated by recent studies on integrated enzyme complexes. Precise cytochemical localization has identified the mitochondria as the site of several such complexes (15, 16, 10, 13, 8). It was observed that the morphological state of the mitochondria determined the capacity of the system to function. The mutual dependence of structural integrity and enzymatic activity indicated that alterations in mitochondria may influence cellular respiration. In the present study, the morphology of the mitochondria is examined in order to determine peculiarities of structure which may affect its enzymatic properties.

EXPERIMENTAL

1. *Morphology*

Within the intact healthy cell the mitochondria are elongated rods. In the convoluted tubules of the kidney they are especially long and traverse the depth of the cell from brush border to basement membrane (Fig. 1). The mitochondria in liver and heart are shorter and thicker and assume no regular arrangement in the cell. When these tissues are homogenized in 17–30 per cent sucrose, the mitochondria are released and retain their rod shape (Fig. 2). With 8.5 per cent sucrose and 0.9 per cent potassium chloride, the rodlet form is replaced by ellipsoids and spheres. By permitting the more dilute solution to flow beneath the cover slip, the alteration in shape of in-

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TABLE I

Oxygen uptake of cyclophorase prepared in different suspending media

Suspending media	μ-Moles of oxygen taken up by the cyclophorase			
	Experiment A	Experiment B	Experiment C	Experiment D
17 per cent sucrose.....	46	70	49	75
8.5 » » »	95	91	91	106
0.9 » » KCl	96	145	102	123

The suspensions were prepared in 0.9 per cent KCl, 8.5 per cent sucrose, and 17.0 per cent sucrose. These preparations were tested for oxidative activity in Warburg flasks which contained 1.5 ml of cyclophorase from liver at the third residue stage (R_3L), 0.3 ml of 0.1 M phosphate buffer of pH 7.3, 0.3 ml of 0.01 M adenosine-5-phosphate, 0.2 ml of 0.02 M magnesium chloride and water to a final volume of 3.0 ml. The experimental vessels contained 0.2 ml of 0.1 M α-ketoglutarate. The center well contained alkali. The gas space was filled with oxygen and the vessels equilibrated in a water bath at 38° C. Oxygen uptake for the first five minutes was presumed to equal that for the subsequent 5 minutes. The oxygen consumption was determined over a period of 30 minutes.

dividual mitochondria from rods to spheres has been repeatedly observed. The rods measure up to 3μ in length and are transformed to spheres of 0.5–1 μ diameter by dilution of the suspending medium.

The cyclophorase activity of preparations made in 17 per cent sucrose, 8.5 per cent sucrose and 0.9 per cent potassium chloride is not similar. Oxygen uptake (Table I) is greatest with the suspensions in saline, least with the 17 per cent sucrose. The spherical mitochondria are associated with considerably greater respiratory activity. There is a clearly manifest difference between units suspended in 8.5 per cent sucrose and in 17 per cent sucrose. It is apparent that under the imposed conditions the spherical form of the mitochondria is not deterrent of oxidation. On the contrary, oxidation is accelerated when the form is thus altered.

The change of shape from rod to sphere is attended by a conspicuous manifestation of internal structure in the units. A sequence of events has been observed in many mitochondria, after their immersion in isotonic potassium chloride and distilled water. The units bend and simultaneously unfurl laterally, until a disc shape is assumed (Figs. 3, 4, 5). The discs have convex and concave surfaces; from the concavity a bubble-like excrescence emerges. This swells at the expense of the "dense" rind, until the structure may resemble a new moon; the residual shadowy crescent represents the site of the pre-existent rodlet, and the sphere is the newly formed element. During this process (Diagram 1), the units may measure up to 3.0 μ in dia-

meter. The composite structure is clearly depicted both by phase microscopy (Fig. 6), and with the selective Fast Green stain (9). If the exposure to deionized water is less than 15 minutes, resuspension in 30 per cent sucrose will cause a reduction in size, with condensation of the crescent and shrinkage of the attached sphere. On the other hand, after prolonged exposure, the internal composite structure disappears (Figs. 7, 8), and the swelling is irreversible. This final condition is attended by loss of cyclophorase activity.

When the mitochondria are treated with capryl alcohol or saturated ammonium sulfate, the structure collapses. The units are irregular, crenated and shrivelled and assume anomalous contours. Subsequent treatment with deionized water or 30 per cent urea causes no swelling. The usual pattern of behavior in dilute and concentrated solutions is obliterated by these transforming agents.

2. *Osmotic behavior*

The swelling and shrinkage of the mitochondria in hypotonic and hypertonic solutions resembles an osmotic system. Experiments were employed to ascertain if the mitochondria are bounded by semipermeable membranes. In one set of experiments aliquots of cyclophorase suspensions were diluted with similar volumes of different solutions, including deionized water, 30 per cent urea, saturated capryl alcohol and saturated ammonium sulfate. After 30 minutes the suspensions were centrifuged for 15 minutes at 4,500 × g

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Fig. 1. A fragment of a renal tubule is seen, in which the refractile basement membrane and the closely packed rod-shaped mitochondria are demonstrated. No nuclei are observed because the intense shearing force of the blendor ruptures them and the cell membranes. The suspending medium is 17 per cent sucrose.
Phase Contrast. B-Minus. 1,400 × Magnification.

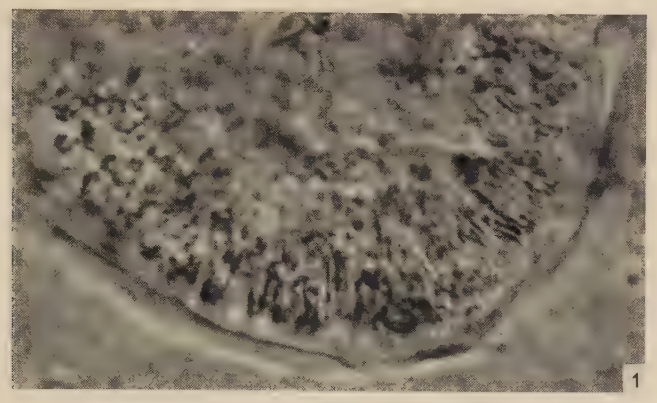
Fig. 2. Several freely floating rod shaped mitochondria are seen. They are suspended in 17 per cent sucrose.
Phase Contrast. B-Minus. 1,500 × Magnification.

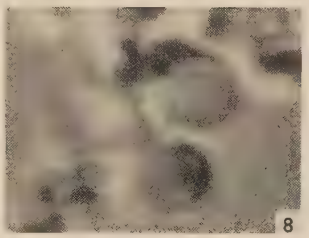
Fig. 3. The rod-shaped mitochondrion is bent as deionized water replaces the hypertonic sucrose by diffusion under the cover slip.
Phase Contrast. B-Minus. 1,500 × Magnification.

Fig. 4. The bent mitochondrion is swelling on its concave margin, with development of a spherical contour.
Phase Contrast. B-Minus. 1,500 × Magnification.

Fig. 5. This mitochondrion is spherical and divided into a light area representing the swollen concavity and a dense crescent, which is the residuum of the original bent rod. The homogenate was prepared in 17 per cent sucrose and resuspended in 0.9 per cent KCl.
Phase Contrast. B-Minus. 1,500 × Magnification.

Fig. 6. The mitochondrion is enspherulated and is composed of an apparently large dense area and a small light region. Rotation of the mitochondrion increased the extent of the light region. Rotation of the mitochondrion increased the extent of the light area, depending on the aspect of the sphere examined. The appearance suggests that the rods not only bend and swell, but also unfurl laterally and become flatter to assume





concave-convex disc shapes. From the concavity of such discs the lighter balloon area develops.
Phase Contrast. B-Minus. 1,500 × Magnification.

Fig. 7. The enspherulated mitochondrion is greatly enlarged (Compare Figs. 5 and 6). It is uniformly homogeneous, and is a full development of the balloon-like excrescence which commences in the concavity of the disc shaped mitochondria. The narrow, crescentric density which occupies a segment of the periphery is a remnant of the original rod. The suspending medium is 17 per cent sucrose, diluted by 20 volumes of deionized water.
Phase Contrast. B-Minus. 1,500 × Magnification.

Fig. 8. The mitochondria were prepared in 0.9 per cent KCl and resuspended in deionized water. Two mitochondria contain residua of the original dense mitochondrial crescents. A third, lowermost in the figure, is a large "ghost like" shadow, which lacks a localized density. Prolonged exposure of the mitochondria to deionized water converts all of them into shadows, without any loss of protein.
Phase Contrast. B-Minus. 1,500 × Magnification.

[1] Photographs 2–8 were taken at 1,500 × magnification and enlarged 3 × from the negative.

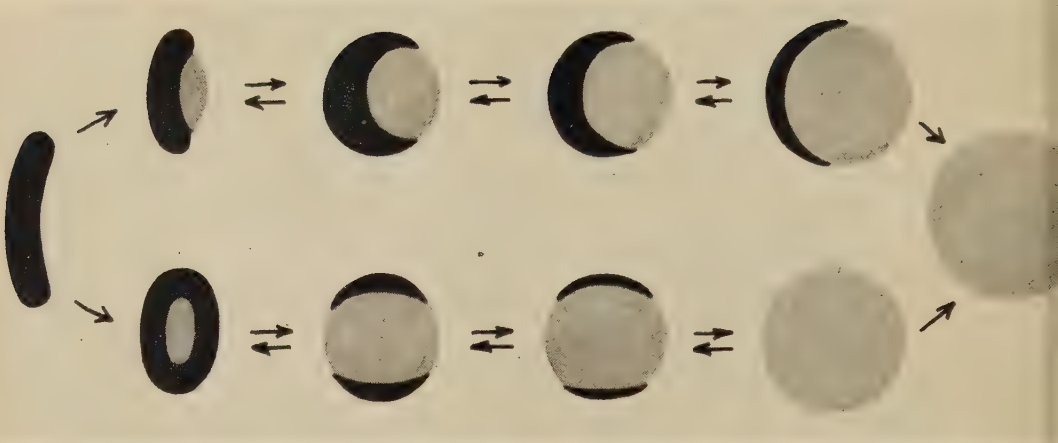


DIAGRAM NUMBER I

The upper row of figures represents the mitochondria in profile, the lower row in facade. The initial alteration from the rod to a concavo-convex disc, with an excresence, is irreversible, as is the final change associated with disappearance of the internal structure. The intermediate four phases depicted are reversible; dilution of the medium causes a shift to the right, concentration initiates a shift to the left. 30 per cent sucrose is used to isolate the rod-shaped mitochondria, and variations of this concentration cause the different forms to appear.

in an angle head[1] refrigerated centrifuge. The supernates from the different specimens were analyzed for their relative contents of protein in solution. It is apparent that neither distilled water nor 30 per cent urea (Table II) causes a release of soluble material into the supernate, although they bring about great swelling of the mitochondria. With capryl alcohol and ammonium sulfate considerable protein is released. The difference in behavior is clearcut and indicates that the mitochondria are not bounded by a membrane which is distended and ruptured by hypotonic solutions with loss of contained protein.

In a second series of experiments cyclophorase preparations were exposed to solutions of radio-active sodium and potassium ions, and then the mitochondria were separated out from suspension by centrifugation. As shown in Table III, the radioactivity per ml of supernate and residue was essentially identical. This was true whether or not oxidation accompanied the exposure of the cyclophorase to the radioactive cations (*cf.* Table IV). The mitochondria (and the included chromosomes) neither exclude the entrance

[1] Centrifugations were performed in an International Refrigerated Centrifuge PR-1, with an angle head of 20 cm diameter.

TABLE II

The quantity of protein and nucleotides released from cyclophorase by different treatments

Reagent or medium	Proteins and nucleotides released, expressed as log $\frac{I_0}{I}$ at 270 mμ		
	Experiment A	Experiment B	Experiment C
0.9 per cent KCl	0.040	0.010	0.033
0.87 per cent NaCl	0.080	0.023	0.032
Deionized water	0.081	0.017	0.035
30 per cent urea	0.072	0.023	0.041
38 °C—1/2 hr	0.060	0.057	0.082
Saturated $(NH_4)_2SO_4$	0.703	0.155	0.114
Saturated capryl alcohol	0.307	0.036	0.116

The cyclophorase was prepared in 0.9 per cent KCl, from kidney and used at the second residue stage (R_2L). Aliquots of 1.0 ml were centrifuged and the residues exposed to the influence of 0.87 per cent sodium chloride, 30 per cent urea, deionized water, and saturated ammonium sulphate solution for 30 minutes. Other aliquots were held at 38° C. for 30 minutes or acted upon by capryl alcohol for the same time. These treated suspensions were centrifuged, the supernates were decanted and aliquots of the supernate dissolved in 10 per cent KOH. With the water clear solutions and distilled water as blank, the absorption at 270 mμ was determined on equivalent amounts of each sample and used as a measure of protein and nucleotides. The supernates of the suspension in 0.9 per cent KCl are the controls, and all other extinction coefficients are compared with this for determination of extracted protein and nucleotide.

TABLE III

The accumulation of Na^{24} (NaCl) and K^{42} (KCl) in the cyclophorase gel

Specimen	Radio-active Na^{24} counts/minute	Radio-active K^{42} counts/minute
Residue		
1	1,545	735
2	1,560	700
Supernate		
1	1,534	775
2	1,545	700

The suspensions of cyclophorase were made in 0.9 per cent KCl. 1.5 ml aliquots of the third washed residue (R_3K) were put into flasks under conditions recorded in Table I, but were maintained for 30 minutes at 0° C. The suspension was then centrifuged at 0° C. for 5 minutes at 500 × g. On the supernates and the residues so obtained, radio-activity measurements were carried out. The supernates and residues were dissolved in 5 per cent KOH and radio-activity measurements made on these solutions.

TABLE IV

The relationship of incorporation of Na^{24} (NaCl) and K^{42} (KCl) and oxidation in cyclophorase suspensions

Experiment	Substrate	Radio-active Na^{24}		Radio-active K^{42}	
		μ moles O_2	counts/minute	μ moles O_2	counts/minute
1	0	1.7	512	2.2	360
	+	60.5	508	59.0	360
2	0	2.0	508	2.5	360
	+	46.5	400	54.4	305
3	0	0	1,790	0.5	1,810
	+	21.6	1,540	1.0	2,670
4	0	0	2,850	0	2,920
	+	22.6	1,870	0	2,730

The cyclophorase was prepared in 0.9 per cent KCl, from kidney and was used as the third washed residue (R_3K). The conditions of oxygen consumption and estimation are those recorded in Table I. At the termination of the oxygen determination the vessels were chilled and the contents washed twice with 50 ml of ice cold 0.9 per cent KCl, which contained 2 ml of M/3 sodium fluoride in experiments 2, 3, and 4. The residues were suspended in 4 ml of 5 per cent KOH and the radioactivity measurements were made on this extract. Background counts throughout these experiments varied between 16 and 33 counts per minute.

of sodium and potassium nor concentrate these ions. This behavior is strikingly different from the selective incorporation of phosphate ions, which depends upon concomitant oxidation. The occurrence or absence of oxidation is without effect upon the behavior of the sodium and potassium ions.

DISCUSSION

Several observers have suggested that mitochondria are bounded by surface membranes (1, 3, 4). The swelling of the mitochondria in dilute solutions is attributed to the semipermeability of such a membrane (13, 18). The present studies cast doubt upon the existence of a membrane with semipermeable properties. The absence of lysis in deionized water and the non-selective penetration of radio-active ions are substantial evidence against the membrane hypothesis. On the other hand, the free penetration of ions, associated with a capacity to swell, is a characteristic of many fibrous gels (5); it is acceptable on all available evidence to regard the mitochondria as tightly coiled or compact gels. A similar conclusion was reached by MacFarlane *et al.* (14) concerning the structure of vaccinia virus particles.

The conversion of mitochondria into "microsomes," with simultaneous

redistribution of the enzymes (8, 12) between the two varieties of particle substantiates this interpretation of the gel-like structure of mitochondria. This is also suggested from the work of Chantrenne (2), who regards the classification of sub-cellular particles as arbitrary and dependent upon the operational effect of gravity. The limitation of swelling short of disintegration and release of protein would in such a structure depend upon the tenacity of the strongest internal cross linkages as suggested by Ferry for gelatin gels. The unrestricted swelling peculiar to plasma membranes is precluded by this arrangement, so that it is inferred that the mitochondria do not simulate the red cells in their composition. The failure of mitochondria to resume their exact rodlet shape when a dilute medium is replaced by a very concentrated one may be due to the irreparable rupture of many cross linkages, which are no longer able to restore the original contours.

The composite structure of mitochondria has been observed by Zollinger (17), although he advances no interpretation of this phenomenon. It is not possible to distinguish the functions of the several parts. But the dissolution of the denser segment is associated with a loss of cyclophorase activity. This close relationship suggests that the denser portion of the mitochondria contains the integrated enzyme complex.

METHODS

The mitochondria were examined in cell free suspensions prepared as described by Green *et al.* (7). The techniques of cytologic examination were previously described (8). Application of transforming agents was made by the methods of Huennekens and Green (11), and the radio-active isotope procedures using sodium and potassium are those employed by Green, Atchley, Nordmann, and Teply (6).

SUMMARY

Mitochondria are visualized as composite structures, in part of which the integrated enzyme systems are concentrated. The behavior of mitochondria in deionized water and in salt solutions is compatible with a gel-like structure and does not require the presence of a semipermeable membrane.

ACKNOWLEDGEMENTS

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LOCALIZATION OF OXIDASES[1]

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INTRODUCTION

The complex of enzymes which implements the reactions of the citric acid cycle has been called cyclophorase (1). Microscopic examination of cyclophorase (2) has indicated that it is essentially a suspension of intact mitochondria, and the enzyme activity has been shown (3) to be related to the structure of these mitochondria.

LePage and Schneider (4) demonstrated that the entire system of glycolytic enzymes could be prepared in the soluble state. Elsewhere it will be shown that the oxidases of glycolysis are present in cyclophorase, although the other enzymes of the Embden-Meyerhof scheme are apparently not associated with the mitochondria. It was therefore of interest to determine whether the pyridinoprotein oxidases of glycolysis occur as soluble enzymes in the intact cells as well as on the mitochondria, or whether the soluble oxidases are derived from the cyclophorase units by some process incident to their preparation. The present communication presents evidence indicating that when a cyclophorase suspension is exposed to the shearing action of the blades of a Waring blendor[4], there is transfer of glycolytic oxidase activity from the particulate elements to soluble proteins simultaneously with the fragmentation of the mitochondria (*cf.* 2).

These observations raised the question whether the phenomenon applies only to the glycolytic oxidases or is true of all the oxidases of cyclophorase.

[1] This investigation was supported in part by a grant from the Commonwealth Fund.

[2] Rockefeller Fellow on leave from the University of Sydney, present address, University of Sydney, Australia.

[3] Williams-Waterman Fellow in Enzyme Chemistry.

[4] Since so much depends on a knowledge of the action of the Waring blendor, a few comments about it seem pertinent at this point. It consists of a glass bowl in which stainless steel blades are rotated at a speed of about 10,000 r. p. m. This causes a shearing action which results in the disintegration of all cells and nuclei in the suspension within 30 seconds. Other subcellular fragments, such as mitochondria, are disintegrated at a slower rate.

A similar investigation of non-glycolytic oxidases of cyclophorase demonstrated that the ratio of activity in the mitochondria to activity in the supernatant from the centrifugation of the homogenate is variable depending upon the enzyme and the organ from which it is derived, as well as the extent of the shearing action to which the preparation has been subjected. Incidentally, it could be shown that another consequence of increased homogenization is conversion of conjugated enzyme, not requiring cozymase (DPN), to dissociated enzyme, requiring the addition of cozymase for activity.[1]

RESULTS

If the oxidases are originally associated with the mitochondria and the soluble enzymes are derived from the mitochondria by some process incident to their preparation, it should be possible to vary the ratio of insoluble to soluble enzyme by modifying the preparative procedure. As shown in Table I, this was found to be the case for some of the oxidases of kidney cyclophorase. For example, when the time of homogenization in the Waring blendor was increased from thirty seconds to ten minutes, the ratio of lactic oxidase activity in sediment to that in supernatant decreased from 0.5 to 0.04. This was true even of succinic oxidase[2], one of the enzymes of the Krebs cycle, the difference being one of degree with more of the total activity initially in the residue. The sediment was a preparation of cyclophorase (R_2K) consisting essentially of mitochondria, and containing some nuclear fragments, whereas the supernatant was essentially equivalent to the combined submicroscopic and supernatant fractions of LePage and Schneider (4). The blending time could not be reduced any further without leaving intact cells in the residue. The question must therefore be left unanswered whether the enzymes are present in the cell in both forms or exist in the cell entirely in association with mitochondria, many of which are broken up immediately either by the shearing action of the blendor blades or by some autolytic process. However, the fragmentation of mitochondria with increased blending time (*cf.* 2) is accompanied by a transfer of enzyme activity from the insoluble to the soluble fraction.

Since the ratio of enzyme activity in sediment to that in supernatant was so

[1] F. M. Huennekens and D. E. Green (Unpublished work reported at American Chemical Society meeting at Atlantic City, September, 1949) showed that under certain conditions cozymase is irreversibly dissociated from various enzymes of cyclophorase to give modified enzymes acting on the same substrates.

[2] This transfer of activity from sediment to supernatant has also been demonstrated for succinic oxidase in Table IV of the first paper of this series (2).

TABLE I

Ratio $\frac{\text{Activity in sediment}}{\text{Activity in supernatant}}$

Time of homogenization min.		Ratio		
		α-Glycerophosphoric oxidase	Lactic oxidase	Succinic oxidase
A	0.5	0.34	0.51	
	2.0	0.16	0.19	
	10.0	0.03	0.04	
B	1.0	0.70		2.13
	5.0	0.11		0.17

Preparation of Enzyme — Demedullated kidneys (2 prs.) were homogenized in a Waring blendor at 0–5° with 180 ml 0.9 per cent KCl containing M/90 NaF. The preparation was kept neutral to bromthymol blue during homogenization. After the minimum time of homogenization, the homogenate was divided into two or three equal parts, one of which was centrifuged directly, whereas the others were homogenized for the additional time desired, frozen salt solution being added to maintain the reduced temperature. After centrifugation at 4,500 g in a refrigerated centrifuge for about 5 minutes, the supernatants (S_1K) were decanted, and the residues were washed with KCl — NaF solution, centrifuged, and suspended in the salt solution (R_2K).

Determination of Enzyme Activity — Enzyme activities were compared in Thunberg tubes at 38°. Suitable aliquots of enzyme solution of suspension were used, and the unit of enzyme activity was taken as that amount of enzyme which will reduce 0.5 ml of 0.1 per cent 2,6-dichlorophenolindophenol in 1,000 secs. All activities were corrected for blanks on the enzyme in the absence of added substrate. Total activity was determined for each fraction by taking into account the aliquot of enzyme used and the total volume. All tubes contained 0.5 ml of 0.1 per cent 2,6-dichlorophenolindophenol, 0.3 ml of 0.1 M phosphate buffer of pH 7.5, enzyme, and water to make a total volume of 2.2 ml. In addition the following were present in the various tubes:

α-Glycerophosphoric oxidase — about 200 μ moles of DL-α-glycerophosphate and about 200 γ of DPN.

Succinic oxidase — about 30 μ moles of succinate.

Lactic oxidase in R_2K — about 450 μ moles of DL-lactate and about 200 γ of DPN.

Lactic oxidase in S_1K — This is assumed to be the sum of conjugated and dissociated lactic oxidases. The former, not requiring DPN, has a pH optimum of 7–7.5, whereas the latter, requiring DPN, has a pH optimum of 9–9.5 (Unpublished observations of Huennekens and Green).

Conjugated lactic oxidase — about 450 μ moles of DL-lactate.

Dissociated lactic oxidase — 0.3 ml of 0.2 M 2-amino-2-methyl-1, 3-propandiol buffer of pH 9.5 in place of the phosphate buffer, about 450 μ moles of DL-lactate and 200 γ of DPN.

much greater for succinic oxidase than for the glycolytic oxidases, it was of interest to determine whether there is a marked difference in the ratio for oxidases of the citric acid cycle. In Table II the distribution of enzyme activity between sediment and supernatant from rabbit kidney and liver is shown for five substrates. Two of the oxidases of the citric acid cycle, α-ketoglutaric and succinic oxidases, were present to a greater extent in the residue, but malic oxidase, another enzyme of the Krebs cycle, had a distribution similar

TABLE II

Distribution of various oxidases of rabbit kidney and liver between sediment and supernatant

Substrate	Ratio $\frac{\text{Activity in sediment}}{\text{Activity in supernatant}}$	
	Kidney	Liver
α-Ketoglutarate	2.67	20.8
Succinate	1.67	2.14
DL-Malate	0.57	0.08
DL-Lactate	0.24	0.09
DL-α-Glycerophosphate	0.20	0.07

The enzyme was prepared by the method indicated in the footnote to Table I, with a homogenization time of one minute. To homogenize one rabbit liver 300 ml of 0.9 per cent KCl containing M/90 NaF was used. Activities were determined similarly. All tubes contained 0.5 ml of 0.1 per cent 2,6-dichlorophenolindophenol, 0.3 ml of 0.1 M phosphate buffer of pH 7.4 (or 0.3 ml of 0.2 M 2-amino-2-methyl-1, 3-propandiol buffer of pH 9.5 for dissociated lactic oxidase in supernatant), enzyme (1.0 ml of S_1K, 0.5 ml of R_2K, 0.2 ml of S_1L, or 0.5 ml of R_2L), and water to make a total volume of 3.5 ml for kidney and 2.7 ml for liver. About 200 γ of DPN was present in all tubes used for testing R_2K or R_2L. The same quantity of DPN was present in tubes used to test S_1K or S_1L for α-ketoglutaric, malic, dissociated lactic, and α-glycerophosphoric oxidases. The following quantities of substrate were used: α-ketoglutarate 30 μ moles, succinate 30 μ moles, DL-malate 200 μ moles, DL-lactate 450 μ moles, and DL-α-glycerophosphate 400 μ moles.

to that of the glycolytic oxidases. Liver and kidney were similar in enzyme distribution, but differed quantitatively. It would therefore appear that all the oxidases can occur in both residue and supernatant, the distribution varying with the enzyme and the organ from which it is derived.

To demonstrate the conversion of insoluble to soluble enzyme by homogenization in the Waring blendor, the usual well-washed preparation of kidney cyclophorase (1) at the third residue stage (R_3K) was homogenized with 0.9 per cent potassium chloride containing M/90 sodium fluoride for five minutes in a Waring blendor at 0–5°. The homogenate was centrifuged at reduced temperature, the supernatant decanted, and the residue washed with the potassium chloride-sodium fluoride solution, and suspended in the salt solution. About 64 per cent of the lactic oxidase activity remaining was in the supernatant, the remainder being in the sediment. It should be noted that the preparation prior to homogenization was leached free of material which would not sediment. Only about 5 per cent of the enzyme in the residue was estimated to be dissociated (requiring cozymase), whereas in the supernatant about 25 per cent was dissociated.

It was thought that if the breakdown of mitochondria is in part the result

TABLE III

Distribution of lactic oxidase from rabbit heart

Fraction	Per cent of total activity	Per cent split
Sediment	43	69
Supernatant	57	100
Insoluble at pH 5.4	28	74
Soluble at pH 5.4	5	100

The enzyme was prepared by homogenization of the minced (scissors) hearts from two rabbits with 200 ml of 0.9 per cent KCl containing 0.01 M NaF for one minute in a Waring blendor at 0–5°. The homogenate was centrifuged in the cold, the supernatant (S_1H) decanted, and the residue washed with KCl — NaF solution, and suspended in the same solution (R_2H). The S_1H was fractionated further by acidification to pH 5,4 at 0–5° by dropwise addition of cold 10 per cent acetic acid. The precipitate was centrifuged, the supernatant decanted and neutralized to bromthymol blue, and the precipitate was washed with KCl — NaF solution at pH 5.4, suspended in the salt solution, and neutralized. Enzyme activities were determined as described in the footnotes to Tables I and II.

of autolytic processes, some other organ such as heart might be more stable than kidney or liver. Cyclophorase was therefore prepared from rabbit heart and the distribution of lactic oxidase between supernatant and residue determined. The supernatant was fractionated further by precipitation at pH 5.4. Microscopic examination of these fractions[1] indicated that there were no intact cells in the residue. The supernatant contained many mitochondria, which probably accounted for most of the activity shown for this fraction in Table III. The acid precipitate from the supernatant contained all the mitochondria and had most of the activity which had not been destroyed by the acid treatment. The supernatant after removal of protein insoluble at pH 5.4 contained no mitochondria and showed only slight activity. However, what enzyme remained was 100 per cent dissociated. The slight amount of conjugated enzyme in the acid precipitate from the supernatant, which was not detected in supernatant before acid precipitation, was well within the error of the analysis, as the acid precipitated represented a sevenfold concentration of the acid-insoluble enzyme in the supernatant. The ratio of activity in sediment to that in supernatant was 0.75, appreciably higher than that for the same enzyme from kidney or liver.

It was also of interest to determine whether increased homogenization in the Waring blendor leads to an increased proportion of dissociated enzyme.

[1] The authors are grateful to Dr. John W. Harman for these observations.

TABLE IV

Effect of homogenization time on the distribution of conjugated and dissociated malic oxidase from rabbit kidney

Homogenization time min.	Per cent of initial activity found				
	Sediment		Supernatant		Total
	Conjugated	Dissociated	Conjugated	Dissociated	
1	39.6	0	17.1	43.3	100.0
5	16.2	7.9	12.0	61.5	97.6

The enzyme was prepared by the procedure described in the footnote to Table I. Activities were determined in Thunberg tubes at 38°. Each tube contained 1.0 ml of enzyme solution or suspension, 0.5 ml of 0.1 per cent 2,6-dichlorophenolindophenol, 0.5 ml of 0.2 M 2-amino-2-methyl-1, 3-propandiol buffer of pH 9.5, 200 μ moles of DL-malate, and water to give a total volume of 3.0 ml. Each enzyme fraction was tested in the presence or absence of about 400 γ of DPN to determine the degree of dissociation, and each velocity was corrected for a blank on the enzyme in the absence of added substrate. Total activity of each fraction was determined as described for Table I. The initial activity was taken as the sum of all the activities after one minute of homogenization. Most of the original activity was recovered after 4 minutes additional homogenization. The pH used was the optimum for dissociated malic oxidase and some of the conjugated enzyme may have been destroyed at this pH (Unpublished observations of F. M. Huennekens).

Malic oxidase was chosen, since it is an enzyme which is quite readily split, but which is sufficiently stable so that the increased proportion of dissociated enzyme cannot be accounted for merely on the basis of destruction of conjugated enzyme without any increase in the total quantity of dissociated enzyme. The data of Table IV demonstrate that when the time of homogenization is increased from one to five minutes, there is not only transfer of enzyme activity to the soluble fraction, but there is also an increase in the degree of dissociation of the enzyme in both the residue and the supernatant.

DISCUSSION

The observation that the proportion of the total oxidase which is found in the mitochondria is variable from enzyme to enzyme, would indicate that either there are two different oxidases acting on the same substrate within the intact cell, or if only one form of any enzyme preexists within the cell, some of the oxidases of cyclophorase must be very readily sheared off from the mitochondria while others are removed only with extreme difficulty. For example, lactic and α-glycerophosphoric oxidases are largely

removed from the mitochondria even under the mildest conditions used, whereas succinic and α-ketoglutaric oxidases are normally present almost entirely in the mitochondria, unless they have been subjected to prolonged homogenization in the Waring blendor. This suggests the possibility that even the mitochondria prepared by the mildest methods are forms already degraded from their state in the intact cell, and that methods have not yet been devised for isolating mitochondria in their original state outside of the intact cell.

SUMMARY

It has been demonstrated that as the time of homogenization of a whole kidney suspension in a Waring blendor is increased, there is transfer of oxidase activity from the sediment to the supernatant, accompanied by splitting out of the prosthetic group of the enzyme in both fractions. This can be correlated with the observation (3) that increased homogenization results in a loss of mitochondria from the residue. Homogenization of a well-washed kidney cyclophorase suspension results in a transfer of some of the activity from the residue to the supernatant. The distribution of enzyme activity between supernatant and sediment varies with the enzyme and the organ from which it is derived. α-ketoglutaric and succinic oxidases are more active in the residue, whereas malic oxidase and the glycolytic oxidases are more active in the supernatant under the usual conditions for the preparation of cyclophorase. The ratio of lactic oxidase activity in sediment to that in supernatant is greater for heart than for kidney or liver.

The authors wish to express their gratitude to Dr. David E. Green for his advice and guidance during the course of this work.

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MITOTIC ACTIVITY IN THE TISSUES OF DEAD MICE, AND IN TISSUES KEPT IN PHYSIOLOGICAL SALT SOLUTIONS

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I. INTRODUCTION

In previous studies on epidermal mitotic activity in mice carried out by Bullough (3, 5) the general conclusion was reached that, while relatively large quantities of glucose and oxygen are necessary for the initiation of a cell division, little or no glucose or oxygen are needed to maintain it once it has started. Thus when a sudden serious sugar lack develops in the skin, due for instance to insulin injections or to ischaemic shock (6), those epidermal mitoses which are already in progress are completed at a normal speed.

The extraordinary insensitivity of the dividing epidermal cell to changes in its environment led to the query whether even the death of the animal could provide a sufficient shock to arrest any mitoses which were at that moment under way. The belief that it could not, and that mitoses already in existence would continue towards completion so long as the tissues remained alive, was strengthened by several odd observations. For instance it was found that no signs of cell division were present in the histological preparations of human epidermis used in the Sheffield Department of Anatomy, and since there had been an interval of several hours between the removal of this epidermis and its fixation, it appeared possible that any mitoses originally present may have been completed. Similarly Parry (10), describing the epidermis of a porpoise which had been some days in transit, remarked on the absence of mitotic activity in the stratum germinativum.

To test this point, the experiments described below were carried out. It is a great pleasure to recall here the generous assistance received from Dr. G. M. Bonser who supplied the mice with mammary carcinomas and spindle cell sarcomas.

[1] Sorby Research Fellow of the Royal Society.

II. MATERIAL AND METHODS

I. THE MICE

In almost all cases the animals used were adult male mice of between 3 and 8 months of age, but the group with spontaneous mammary carcinomas were females of over 12 months of age. The males all belonged either to the Kreyberg's white label or the Strong's CBA strain, while the females belonged either to the Kreyberg's white label, the R III, or the Strong's A strain. No differences in reaction were found between the tissues of these various types of mouse.

All the animals were fully fed on a diet of commercial rat cake, flaked maize, dog biscuit, and cod liver oil. The daily food was always given between 09.00 and 10.00 hr., a routine that was strictly observed in order to establish a steady diurnal rhythm of waking and sleeping and therefore also of mitotic activity. The temperature of the animal room was maintained at 20° C, and the experiments were carried out during the spring and summer.

II. METHODS OF KILLING AND OF FIXATION

In order that the tissues should have the greatest chance of survival for several hours after the death of the mice, the animals were not killed by anaesthetic but by a sharp pull on the neck. The bodies were then left lying either at room temperature or in an oven as described below, and pieces of tissue were removed at hourly intervals. These tissue fragments were all fixed in Bouin's alcoholic fluid, embedded in paraffin wax or, in the case of the skin, in ester wax, and sectioned at 7 μ. In all cases the stains used were Ehrlich's haematoxylin and eosin.

In the later experiments in which fragments of epidermis were placed in physiological salt solutions, the same methods of fixation and sectioning were used. The technique whereby these fragments were maintained alive is described in the course of the observations.

III. METHODS OF ASSESSING MITOTIC ACTIVITY

Two methods were devised for determining the mitosis rate. The first, used in the case of the epidermis, involved counting all mitoses in unit section lengths of 1 cm, and from each sample of epidermis 10 such counts were made and the average taken. The second, used for solid tissues, was similar except that the counts were made on unit section areas of either 0.05 or 0.1 mm^2. In either case the figures obtained from each group of samples were analysed for the mean and standard error according to a method recommended by Simpson and Roe (11).

III. OBSERVATIONS

1. *Mitosis after death*

I. EAR EPIDERMIS

The preliminary experiments involved the killing of a group of 5 adult male mice, and the subsequent removal of small pieces of their ears. The

TABLE I

Average numbers of mitoses present per unit length (1 cm) of sections (7 μ thick) of ear epidermis from a group of 5 male mice killed at 13.00 hr. and maintained at 20° C

Time of day	Numbers of mitoses	Phases of division
13.00	6.2±0.19	PMAT
14.00	1.8±0.30	MAT
15.00	0.6±0.16	AT
16.00	0.3±0.13	T
17.00	0.2±0.11	T
18.00	0.3±0.13	T

In the phases of division P represents the prophase, M the metaphase, A the anaphase, and T the telophase.

mice were killed at 13.00 hr., a time when they were normally asleep, in order that their epidermal mitosis rate should be high (2). Immediately after death an earclip was taken and fixed, and the bodies were then left lying on a bench in a room maintained at 20° C. Further earclips were taken and fixed at hourly intervals until 18.00 hr. The results of this experiment are shown in Table I. In making the counts mitoses in every stage of division were included, and the stages most commonly seen at each hour were also recorded.

In the first place these results demonstrate clearly that the great majority of the mitoses present in the epidermis at the time of death were able to pass to completion before the cells themselves died. In the second place, since it is already known that the time taken for the completion of an epidermal mitosis in an adult male mouse is about 2½ hr. (5), it is evident that their speed was not significantly affected. However, it is also evident from the observed phases of division that no new mitoses were able to develop after 13.00 hr.

The fact that a few telophases continued to be seen after 16.00 hr. suggested that they had been immobilised, a conclusion which may be taken to indicate either that cell death occurred about that time or that some other factor intervened. It was considered possible that the relatively low temperature of 20° C might be partly responsible, and, in an attempt to eliminate all mitoses from the epidermis, the experiment was repeated as before except that the bodies were kept in an oven at 37° C, which is the approximate blood temperature. The results are shown in Table II.

This experiment provided full confirmation for the conclusions reached

TABLE II

Average numbers of mitoses present per unit length (1 cm) of sections (7 μ thick) of ear epidermis from groups each of 5 male mice killed at 13.00 hr. and maintained at 37° C

Time of day	Ears attached to bodies		Ears separated	
	Numbers of mitoses	Phases of division	Numbers of mitoses	Phases of division
13.00	6.1±0.32	PMAT	6.5±0.43	PMAT
14.00	2.4±0.27	MAT	2.8±0.34	MAT
15.00	1.4±0.28	AT	1.4±0.35	AT
16.00	0.9±0.22	T	1.0±0.28	T
17.00	0.9±0.22	T	1.0±0.27	T
18.00	0.8±0.23	T	1.0±0.29	T

In the phases of division P represents the prophase, M the metaphase, A the anaphase, and T the telophase.

above, but the new conditions did not eliminate the last few telophases remaining after 16.00 hr. Indeed there were actually more of these residual mitoses at 37° C than there were at 20° C, although the significance of this difference is doubtful.

As a final variation of this experiment, mice were killed at 13.00 hr., and their ears were cut off. The ears were placed in an oven at 37° C, no precautions being taken to prevent drying, and pieces were removed as before. The results are also shown in Table II, and they are almost identical with the earlier results. This is a particularly interesting observation since it opens the way for a variety of experiments, the first of which is recorded later in this paper.

II. SMALL INTESTINE

To discover whether these reactions are peculiar to the epidermis, the progress of mitosis after the death of the mouse was also studied in the small intestine. The first region examined was the proliferating zone of the intestinal mucosa. The mice used were the 10 adult males on which the results shown in table 2 are based. When they were killed at 13.00 hr., pieces of intestine were removed and fixed immediately. Then the bodies were placed in an oven at 37° C, and, to prevent desiccation, the openings made in the body walls were plugged with wads of cotton wool soaked in Locke's solution. At hourly intervals further pieces of small intestine were removed, and fresh wads of cotton wool were inserted. The results are shown in Table III, and it

TABLE III

Average numbers of mitoses present per unit area (0.05 mm^2) of sections (7 μ thick) of regions of the wall of the small intestine from a group of 10 male mice killed at 13.00 hr. and maintained at 37° C

Time of day	Proliferating zone of mucosa		Centres of lymph nodules	
	Numbers of mitoses	Phases of division	Numbers of mitoses	Phases of division
13.00	7.3±0.20	PMAT	4.6±0.17	PMAT
14.00	3.9±0.18	MAT	0.2±0.08	T
15.00	3.4±0.27	MAT	0	—
16.00	3.5±0.22	MAT	0	—
17.00	3.3±0.23	MAT	0	—
18.00	3.3±0.23	N	0	—

In the phases of division P represents the prophase, M the metaphase, A the anaphase, T the telophase, and N unclassifiable necrotic mitoses.

can be seen that they are not nearly so striking as those obtained with the epidermis.

While in the first hour many mitoses must have passed to completion, throughout the next four hours all the remaining mitoses were static. A study of the sections suggested that the reason lay in the early death of the cells concerned, and by 18.00 hr. most of the mitoses were disintegrating. Even at 14.00 hr. there were signs that the mucosa was breaking down, perhaps through the action of the digestive enzymes in the intestinal lumen. By 15.00 hr. the breakdown was proceeding rapidly, and at 17.00 and 18.00 hr. examples were discovered in which the underlying connective tissue was exposed. Possibly if the intestine had been removed at death and its contents washed out, the dividing cells might have survived longer and more of them might have reached the resting phase.

The other regions of the intestinal wall to be examined were the proliferating centres of the intestinal lymph nodules, which normally maintain a high rate of cell division both in waking and in sleeping (2). The results of this survey are also shown in Table III, and it can be seen that in this tissue almost all the mitoses passed to completion during the first hour after death. After the second hour no more mitoses were visible. Thus it appears that these cells not only develop a constantly high rate of mitosis, but that in addition each division is completed in an unusually short space of time. Clearly it was this latter peculiarity that enabled the cells to complete their divisions before they themselves died.

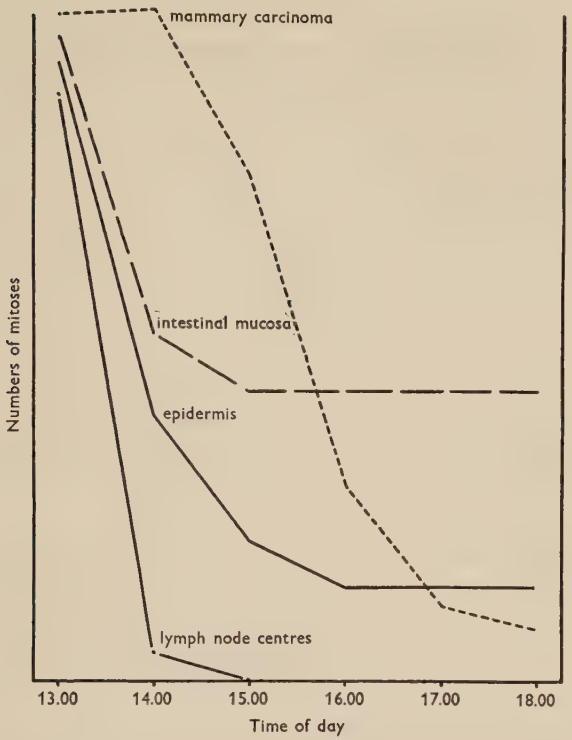

Fig. 1. The post-mortem changes in the numbers of mitoses visible in the tissues of mice killed at 13.00 hr.

III. TUMOURS

After these observations on normal tissues it seemed of interest to examine conditions in abnormal cells. For this purpose studies were made on spontaneous mammary carcinomas in old female mice and on spindle cell sarcomas implanted into adult males. The experiments were carried out as before, the mice being killed at 13.00 hr. Small pieces of the tumours were removed at that time and at hourly intervals afterwards. Meanwhile the bodies were kept in an oven at 37° C, and the cuts in the body walls were covered with pads of cotton wool soaked in Locke's solution. The results are shown in Table IV.

The reactions of these two tumours to the stoppage of the blood supply were almost exactly similar. However, they differed markedly from anything so far described in that new prophases continued to be formed for at least two hours post-mortem. This continued formation of new mitoses is not

TABLE IV

Average numbers of mitoses present per unit area (0.1 mm^2) of sections (7 μ thick) of tumours in groups of 5 female and of 5 male mice

Time of day	Spontaneous mammary carcinoma		Implanted sarcoma	
	Numbers of mitoses	Phases of division	Numbers of mitoses	Phases of division
13.00	3.5±0.37	PMAT	4.2±0.31	PMAT
14.00	3.6±0.42	PMAT	4.0±0.39	PMAT
15.00	2.7±0.43	PMAT	1.8±0.15	PMAT
16.00	1.1±0.18	MAT	1.2±0.18	MAT
17.00	0.4±0.12	T	0.6±0.13	T
18.00	0.3±0.11	T	0.2±0.07	T

In the phases of division P represents the prophase, M the metaphase, A the anaphase, and T the telophase.

obvious from the data recorded in the table; it could equally be argued that the whole process of mitosis had been arrested until after 14.00 hr. However, in the sections themselves the progress of cell division could be followed for while at 13.00 hr. there was a preponderance of prophases and metaphases, by 14.00 hr. there was a preponderance of anaphases and telophases. It seems that the numbers of new mitoses formed between 13.00 and 14.00 hr. were lower than they would have been in the living animal, a fact which is further reflected in the reduced numbers of mitoses visible at 15.00 hr. However, a few apparently newly-formed prophases were still to be seen at 16.00 hr., and even at 18.00 hr. a very few prophases were present in one of the spindle cell sarcomas.

Thus it was evident that cell division in these tumours resembled that in normal tissues in that it could be completed after the stoppage of the blood supply, but differed in that it could also be started in these circumstances.

2. *Epidermal mitosis in physiological salt solutions*

I. INTRODUCTION

These results suggested further ways in which mitotic activity in mouse tissues could be studied. In particular it appeared that pieces of epidermis maintained alive might be made to give information which could otherwise not easily be obtained. As a preliminary to such a study the following experiments were performed.

Adult male mice were killed as before, and their ears were removed and cut into small fragments (about 5 × 2.5 mm in size). These were immediately immersed in salt solution held in hollowed glass blocks, each of which contained 4 ml of liquid and an air bubble of about 1 ml, and was covered by a glass plate. One or sometimes two fragments of ear were placed in each block, and the blocks were clamped on to a mechanical shaker in a room maintained at 20° C. For the first few minutes there was a tendency for the ear fragments, because of their greasy surfaces, to float on the liquid, but the constant shaking soon caused them to sink.

II. EPIDERMAL MITOSIS IN LOCKE'S SOLUTION

The first salt solution used was a modification of Locke's solution from which glucose was omitted and its place taken by extra NaCl. Ten mice were killed at 13.00 hr., and their ears were detached and cut into small pieces by means of a razor-sharp scalpel. One ear fragment from each mouse was fixed immediately, and the others were immersed in the solution. At hourly intervals further pieces of ear were removed for fixation.

The results obtained differed slightly from those described above, probably because the epidermis remained alive during the whole period of 5 hr. Thus there was ample time available for the completion of those mitoses which were in progress when the mice were killed, and after three or four hours, some of the ear fragments were actually found to lack any sign of mitotic activity. The results obtained also differed in another particular, for, after two or three hours, a few of the ear fragments were found to contain new mitoses. The number of these new mitoses was very small, but it was sufficient to account for the fact that the average figures given in Table V do not show a decline to zero by 17.00 or 18.00 hr. In order to differentiate between the original and the new mitoses in the phases of division shown in this table, the former are indicated by capital letters and the latter by small letters.

It can be concluded that in Locke's solution modified by the removal of glucose the ear epidermis remains alive for at least 5 hr., that the mitoses originally present are completed normally, and that a very few new mitoses develop.

For comparison, fragments of ear from the same 10 mice were simultaneously maintained in normal Locke's solution which contains 0.2 per cent of glucose. The results obtained are also shown in Table V, and it is clear that the presence of sugar greatly favoured the development of new mitoses. In

TABLE V

Average numbers of mitoses present per unit length (1 cm) of sections (7 μ thick) of the epidermis of ear fragments taken from 10 male mice and maintained in saline solutions

Time of day	In Locke's solution without glucose		In Locke's solution containing glucose	
	Numbers of mitoses	Phases of division	Numbers of mitoses	Phases of division
13.00	7.3±0.24	PMAT	7.3±0.24	PMAT
14.00	3.3±0.38	MAT	4.3±0.42	MAT
15.00	1.9±0.31	AT	5.4±0.41	pmAT
16.00	1.5±0.19	pm T	4.7±0.33	pmaT
17.00	0.7±0.24	pmat	2.7±0.26	pmat
18.00	0.6±0.28	pmat	1.7±0.37	pmat

In the phases of division P represents the prophase, M the metaphase, A the anaphase, and T the telophase; the capital letters refer to mitoses in progress at the time of the animal's death while the small letters refer to newly developed mitoses.

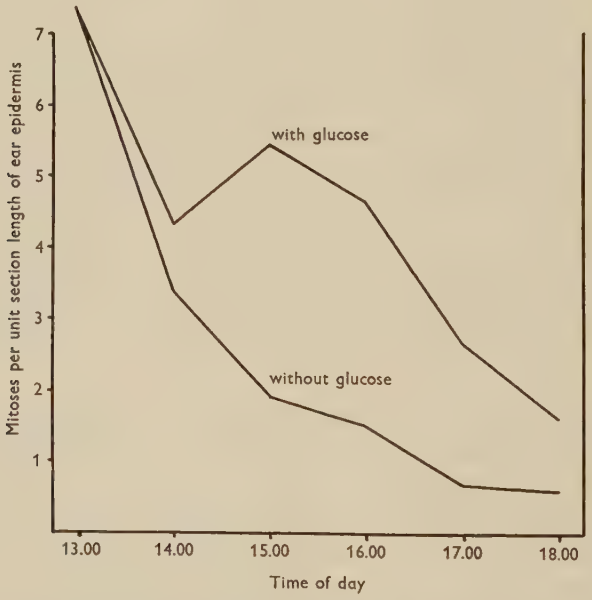

Fig. 2. The changes in the numbers of mitoses visible in fragments of ear epidermis removed from mice killed at 13.00 hr. and maintained in physiological salt solutions.

this experiment the new prophases appeared earlier and in far greater numbers, a result which confirms the importance of sugar in epidermal mitotic activity.

IV. DISCUSSION

These results indicate that in a number of tissues, normal as well as abnormal, any mitoses which are in progress when the blood circulation ceases are able to continue towards completion in an apparently normal manner. The ability of a dividing cell to reach the resting phase once more appears to depend partly on its speed and partly on the length of the interval before the tissue itself dies. Thus the high speed at which each mitosis is completed in the proliferating centres of the intestinal lymph nodules ensures that all these divisions are able to pass to completion, while in the intestinal mucosa the death of the cells takes place so rapidly that large numbers of mitoses are arrested.

An intermediate situation occurs in the epidermis where all but a few mitoses are usually completed. However, when the lives of these cells are prolonged by immersion in a physiological salt solution the completion of all mitoses becomes possible. Also in these circumstances a few new mitoses may develop, but this process is not active unless glucose is present.

As far as the present information goes, new mitoses are never formed in any normal tissue not kept in a culture solution. The stoppage of the blood supply causes an immediate stoppage of mitosis formation, but the two tumours examined were peculiar in that they did develop new mitoses during a period of at least two hours after the death of the mice.

The primary interest of these results lies in the confirmation which they afford for views already expressed on the physiology of cell division, particularly in mammalian epidermis (3, 4, 5). It has been shown that a cell is unable to begin division unless sufficient quantities of glucose, phosphate, and oxygen are available, and it has been suggested that the onset of a mitosis is characterised by the aerobic destruction of glucose to provide energy. If this theory is correct, it appears that this energy is not all used immediately, but that some is stored to be released as required during the course of the division. Thus an explanation may be provided for the fact that, while no epidermal mitosis can develop in the absence of oxygen (9) or glucose, any mitosis which has progressed far enough to be recognisable can proceed quite independently of both oxygen and glucose (Bullough and Johnson, unpublished). The present results are clearly in agreement, and demonstrate in a striking manner the extraordinary independence of the dividing cell.

Extending this argument, it now appears that tumour cells differ in that they can begin division in anaerobic conditions. Anaerobic mitosis is, of course, a well known possibility in certain cells, and it can be mentioned that the eggs of *Fundulus* (7) and of the frog (1) are able to develop normally for many hours in the total absence of oxygen. In such cases it seems possible that during an emergency some process of anaerobic glycolysis is able to provide sufficient quantities of energy, whereas in other cells, such as the sea urchin egg (8) and mouse epidermis, this is not so. Experiments on this point are now under way.

One interesting result of the present work has been the development of an extremely simple technique whereby new and more critical experiments can be planned for testing these various theories. A considerable amount has already been learned about epidermal mitosis *in vivo*, and more may now be expected from complementary investigations *in vitro*.

V. SUMMARY

1. It is shown that in a variety of normal mouse tissues those mitoses which are in progress when the animal is killed continue towards completion for as long as the tissue itself remains alive. During this interval new mitoses developed in tumour cells, but they did not do so in any of the normal tissues examined.

2. This is in agreement with a theory, previously put forward, that glucose and oxygen, the most critical substances in mitosis, are necessary only at the very beginning of the process. Once started, mitosis may continue to completion in the absence of either.

3. When maintained in Locke's solution the length of life of the ear epidermis is greatly prolonged. In these circumstances the presence of glucose is shown to act as a stimulus to the production of new mitoses.

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A CYTOCHEMICAL ANALYSIS OF THE FUNCTIONAL INTERRELATIONS OF VARIOUS CELL STRUCTURES IN ARVELIUS ALBOPUNCTATUS (DE GEER)

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I. INTRODUCTION

In recent years, cytochemistry has made notable advances, and it is becoming more and more useful in our analysis of the functional interrelations of various structures in the cell. Thus the growth of the cytoplasm, the role of the nucleolus in the cell, and the relation of the chromosome to the nucleus as a whole are some of the problems that are engaging various investigators. In the following pages we report on a cytochemical analysis of certain conditions encountered in the pentatomid insect *Arvelius albopunctatus* which involve several of the questions just mentioned. Peculiarly advantageous for our purposes is the fact that the testis of this species normally contains germ cells which, in sharply demarcated regions or lobes, show constant differences in the volume of several of the cell constituents — the nucleus, the nucleolus and the cytoplasm.

It has been known since Montgomery's investigations (30) that in the pentatomid *Euschistus* certain lobes of every testis contain cells which differ quite regularly in size from those of the other lobes. Bowen (3) later found such "polymegaly" also in fifteen other species of the Subfamily *Pentatominae,* and the investigations of the present senior author make it likely that the majority of species in that Subfamily of insects show this regular variation of cell size. In all known cases there are two lobes of the testis in which the spermatocytes are distinctly larger than common. The spermatocytes of the remaining lobes conform to the more usual or normal dimensions, although some species have a single lobe which carries cells that are smaller than normal. In a few species these size differences are especially striking, and the outstanding example among these is *Arvelius albopunctatus.*

TABLE I

Size relations in 3 types of spermatocytes.

Type of cell	Mean cellular volume in cubic microns	Mean nuclear volume in cubic microns	Mean cytoplasmic volume in cubic microns
Large sized spermatocyte "confused stage"	9,874 ± 967	1,596 ± 108	8,278 ± 801
Normal sized spermatocyte "confused stage"	2,953 ± 290	408 ± 43	2,545 ± 245
Small sized spermatocyte "confused stage"	1,376 ± 150	230 ± 32	1,146 ± 126

But in all these cases the meiotic and sperm forming processes are apparently quite normal.

It may be remarked that in its manifestations, polymegaly is quite distinct from the singular developments that are encountered in the so-called "harlequin" lobe. The latter is found in relatively few species, and its cells are characterized by rather definite departures from the normal meiotic process. In all the cases so far studied, only a single lobe is thus affected, and it is probable that the abnormal behavior of the spindles and the chromosomes is due to an irregular timing of the mitotic steps in the spermatocytes (37, 38, 39). In contrast, the polymegaly with which we are concerned in the present paper involves no abnormalities of meiotic behavior, and the cytological departures from the normal are expressed only in unusual size relations. The cytological and cytochemical analysis of the regular size differences in the nucleus, nucleolus and cytoplasm led to a clearer conception of the functional relations between these different constituents of the cells. While the increase of nuclear, nucleolar and cytoplasmic volume seemed to be intimately linked with each other and correlated with an increase in protein and ribosenucleic acid, their quantitative changes were in no way reflected in any alteration of the amount of DNA in the nucleus. From the photometric microscopic measurements reported in the following pages it is evident that the amounts of protein and ribosenucleic acid (RNA) increase during the growth of the cells from about 2 to 8 times (dependent on the cell structure analyzed) in contrast to the desoxyribosenucleic acid (DNA), which does not change in quantity.

Although the determination of the relationship between nucleus, nucleolus

and cytoplasm was the main purpose of this study, the cytological and cytochemical findings will be presented separately for each of the cellular constituents, followed by a general discussion of the results.

II. MATERIAL AND METHODS

Eight males of *Arvelius albopunctatus* were collected in the vicinity of Turrialba, Costa Rica, during April, May and June of 1944 and 1948. All the specimen were in good condition, and the testes were dissected out and fixed within 1 to 4 hours of the time of capture. All of them were fixed in Sanfelice for 4 to 5 hours and washed in water for at least 12 hours before dehydration and imbedding. Sections were cut at various thicknesses, according to the requirements of the investigation. The photometric measurements were made by the method originally described by Pollister and Ris (33). A description of the apparatus in its present form and a discussion of the photometric microscopic methods is given by Leuchtenberger (28) and Swift (46).

In the present study the desoxyribosenucleic acid was estimated after the Feulgen reaction from measurements of the absorption of the 546 millimicrons line from a Mercury lamp by a Wratten 62 filter. In each case the transmission through a central cylinder of the nucleus was measured and the DNA per nucleus expressed in arbitrary units on the basis of computations as described by Swift (46).[1]

For the estimation of the proteins in nuclei and nucleoli the Millon reaction was carried out as previously described by Pollister and Leuchtenberger (32). The color developed by the Millon reaction was measured as absorption of the strong 365–6 millimicron lines isolated from a G. E. AH_4 mercury vapor lamp by a Corning Glass filter No. 5840. The amounts of proteins in the tables are also expressed in arbitrary units and obtained by multiplying the concentration of the color times the nuclear volumes; that is $\frac{E}{D} \times V$ where E = exctinction, D = thickness, V = volume.

An estimation of the basic proteins in the nucleolus was attempted by staining slides with a 0.1 per cent watery solution of Fast Green (Fast Green FCF, National Aniline Division, total dye content 96 per cent) at a pH of 1.2 for 1 hour and differentiating the slides for 24 hours in 100 per cent alcohol. Such a procedure seemed justified, since Chapman, Greenberg and Schmidt (9) and others have demonstrated that there exists a stoichiometrical relation between the amount of free basic groups in various proteins and their capacity to bind acidic dyes at a low pH. The differentiation in alcohol for 24 hours was made in order to reduce possible adsorption of the dye to a minimum and to obtain chemical staining according to Michaelis.

[1] The formula used is the one employed by Swift (46), as follows:

$$A = \frac{E \times \pi r^2_{\text{plug}} \times 100}{\text{per cent Volume}}$$

where A = Amount in arbitrary units; E = Extinction of plug; πr^2 = Area of cross-section of plug measured; per cent Volume $= \frac{\text{Volume of plug} \times 100}{\text{Volume of whole sphere}}$.

Fig. 1. Semidiagrammatic drawing of *Arvelius testis*. Ripe sperms leave testis through sperm duct at upper end; spermatogonia are at lower end. Lobes 3 and 5 contain large spermatocytes; lobe 4 contains small spermatocytes.

The Fast Green in the nucleoli was measured as absorption of the red light isolated from a zirconium lamp by means of a Wratten 26 Filter.

For the estimation of the ribosenucleic acid in the nucleoli the Azure stain (Azure A) was used according to Flax and Pollister (14). Unfortunately, it was not possible

to test the ribosenucleic acid in combination with ribonuclease, as had been done previously in corn nucleoli (32) since the ribonuclease did not seem to have any detectable effect on the Sanfelice fixed material. Therefore slides in which the nucleic acids were extracted with hot trichloracetic acid and stained with Azure were used as controls. The low staining intensity of Azure A in the nucleoli suggests that Azure A may stain only a fraction of the total ribosenucleic acid of the nucleoli in *Arvelius*, which seems to be in agreement with the observation of Flax and Pollister. In spite of this restriction, it seemed justified to compare relative amounts of RNA by this method in the large and small-sized nucleoli, since nucleoli of the same type of cells within one slide were compared with each other. The Azure stain in the nucleoli was measured as absorption of the red light isolated from a zirconium lamp by means of a Wratten 26 filter.

It should be emphasized that the comparative measurements for a given experiment were usually made on a single slide. Since only a single testis was mounted on each slide, the values obtained for different size classes of the various cell elements are based on material in which there is no possibility of variation in treatment. Indeed, in some cases the different measurements were made on cells of a single section and, sometimes, even in the same field of vision. Throughout the paper the values are given in arbitrary units.

In an addendum to a recent paper by Ris and Mirsky (35) the last-named author has questioned the reliability of the methods used in this laboratory, making special reference to the cytochemical measurements in different tissues of Tradescantia by Schrader and Leuchtenberger (40). He points out that when, after ordinary fixation such as Carnoy, photometric measurements are made of the DNA in nuclei differing markedly in structure — such as erythrocyte and liver nuclei — errors as high as 34 per cent are possible. This criticism is, of course, not applicable to our measurements in Tradescantia for, in comparing various tissues of that plant, all the nuclei used were, with one exception,[1] in early prophase and interphase stages. Such measurements are possible, as Ris and Mirsky themselves state (35, p. 143) "with an error of about 10 per cent" and since interphase nuclei of the root tip, leaf and bud tapetum differ in DNA content by from 60 per cent to more than 100 per cent, our conclusion that the different Tradescantia tissues examined by us did not carry identical amounts of DNA seems amply warranted.[2]

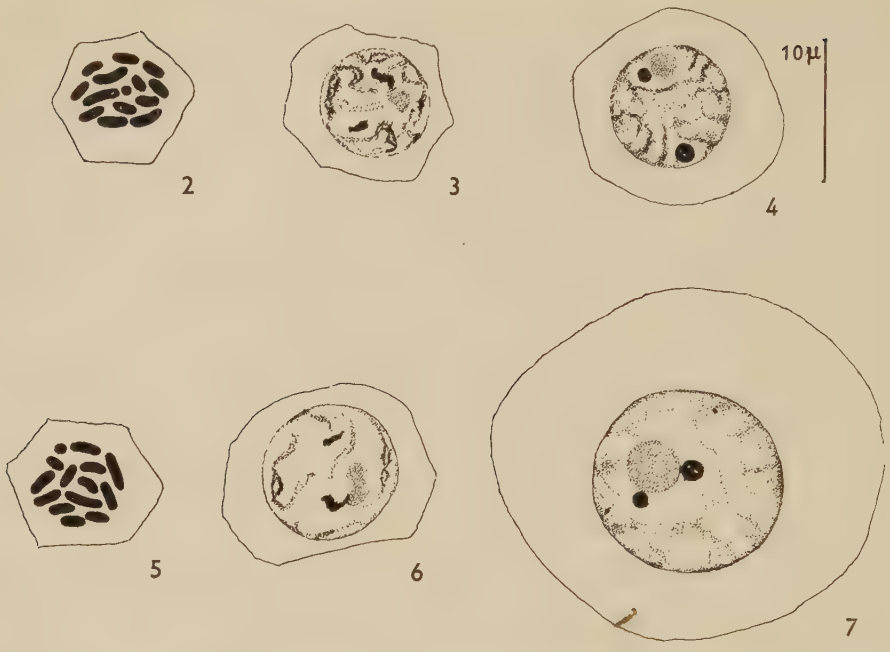
III. THE NUCLEUS

1. *Structural basis*

The testis of *Arvelius* has 6 lobes. These are numbered, starting from the side at which the sperm duct makes its exit. Lobes Nos. 1, 2 and 6 carry cells which conform to the more common size observed in the testis of other

[1] This exception consisted of pachytene stages in bud tissue. The difficulty of comparing such stages with interphases was specifically noted.

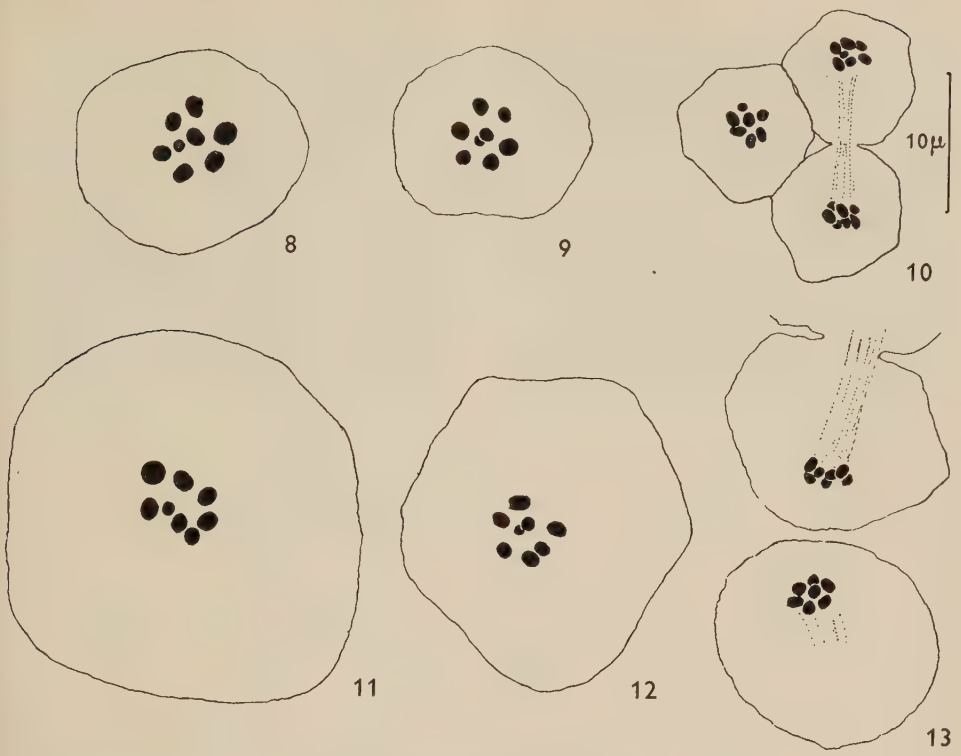
[2] In attributing to Mirsky and Ris a complete agreement with the Boivin-Vendrely rule that the amount of DNA is the same in all diploid cells of a given species, we (40) overlooked their report that beef tissues constitute an exception (29).



Figs. 2 & 5. Spermatogonial metaphase (of final spermatogonial division) in small cell lobe (Fig. 2) and large cell lobe (Fig. 5). Cell size and chromosome size are alike in the two cells.
Figs. 3 & 6. Diplotene stage in small (Fig. 3) and large (Fig. 6) spermatocyte. Latter is already distinctly larger.
Figs. 4 & 7. Confused stage in small (Fig. 4) and large (Fig. 7) spermatocyte. Sex chromosomes approximately of same size in the two cell types, but cytoplasm, nucleus, and nucleolus much larger in large spermatocyte.

pentatomids; lobes 3 and 5 are characterized by spermatocytes and spermatids of abnormally large dimensions; and lobe 4 has cells somewhat smaller than normal. The number of cells and diameter of the lobes are, in general, inversely proportional to these cell sizes; the 4th lobe is greatest in diameter and contains by far the most cells, and the 3rd and 5th lobes are the smallest and carry the fewest cells (Fig. 1).

The spermatogenesis of the Pentatomidae follows a rather uniform course in the many species that have been studied. The premeiotic interphase is usually followed by a prochromosome stage, and this in turn by the leptotene, synaptotene (synezesis), pachytene, diplotene, confused and diakinetic stages, terminating finally in the first spermatocyte metaphase. It is in the transition from the diplotene (Figs. 3 and 6) to the confused stage (Figs. 4 and 7) that the greatest increase in nuclear, nucleolar and cytoplasmic



Figs. 8 & 11. First metaphase in small (Fig. 8) and large (Fig. 11) spermatocyte.
Figs. 9 & 12. Second metaphase in small (Fig. 9) and large (Fig. 12) spermatocyte.
Figs. 10 & 13. Telophase of second spermatocyte division in small (Fig. 10) and large (Fig. 13) spermatocyte. There is no difference in size of the chromosomes in the small and large spermatocytes.

dimensions occurs. In the nucleus of the confused stage the chromosomes temporarily become diffuse in outline and approach a state of dispersion which frequently appears to be almost as extreme as that of the typical interphase (Figs. 4, 7, 14, 15). The two meiotic or spermatocyte divisions (Figs. 8–13) culminate in the formation of spermatids. The nuclei of these, regardless whether in small, normal or large cells, are all identical in size immediately after the membrane has formed around the telophase group of chromosomes succeeding the second spermatocyte division (Figs. 16, 18). But thereafter a differential growth of these spermatid nuclei occurs, which once more reflects the different sizes of the earlier confused stage nuclei in the different lobes (Figs. 17, 19, 20). As Bowen (3) has reported, the com-

plicated maneuvers which attend the transformation of the spermatid to the finished sperm are essentially alike in all three size classes.

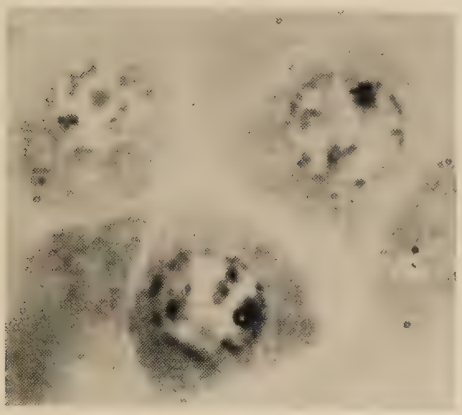
A more detailed examination of the nuclear size differences is indicated. In all six lobes of the testis the spermatogonial nuclei of a given cell generation agree closely in size (Figs. 2, 5). This similarity extends to the interphase nuclei of the first spermatocyte, and it is not until the leptotene stage has been completed that the first indications of a differential nuclear growth in the different lobes become apparent. The maximum size in all lobes is attained early in the confused stage. But in the giant nuclei of the 3rd and 5th lobes the chromosome threads are more diffusely distributed or the chromatin more diluted, as it were, than in the normal nuclei in the other lobes (Figs. 14, 15), and when the metaphase chromosomes finally appear in the first metaphase, they do not reflect in their size the volume of the large nuclei in which they evolve (Figs. 8, 11). The meiotic divisions are very much alike in all the different lobes, and the varying amounts of cytoplasm that characterize the cells of the different lobes do not visibly affect the meiotic process. Indeed, when after the second division a nuclear membrane first reappears around the telophase chromosomes, all the nuclei (the spermatid nuclei) are once more identical in size, just as were the nuclei in the spermatogonial and meiotic interphases and leptotene stages (Figs. 16, 18).

But it is to be noted that the differential size growth of the prophase nuclei is now repeated in these spermatid nuclei. Those of the 3rd and 5th lobes become very much larger than the others and then again show a more dilute distribution of the chromatin (Figs. 17, 19, 20). This growth in size stops at approximately the time when the nuclei show the first traces of elongation. According to Bowen (3) the giant nuclei finally evolve spermhead nuclei that are no larger than those formed from normal nuclei. The factors involved in these final steps in sperm formation will be dealt with in a future publication.

It should be emphasized that the growth processes which result in such great size differences in the different lobes do not affect the meiotic phenomena in the chromosomes in any perceptible way. The formation of bivalents and the two meiotic divisions follow orthodox lines and are identical in all the three sizes of spermatocytes (Figs. 2–13).

2. *Photometric measurements of nucleus*

A. Nuclear size and its relation to DNA. It has been pointed out by Montgomery (30), Bowen (3) and Schrader (39) that, although the size of the



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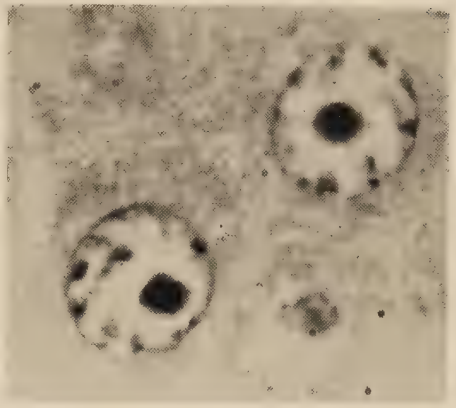

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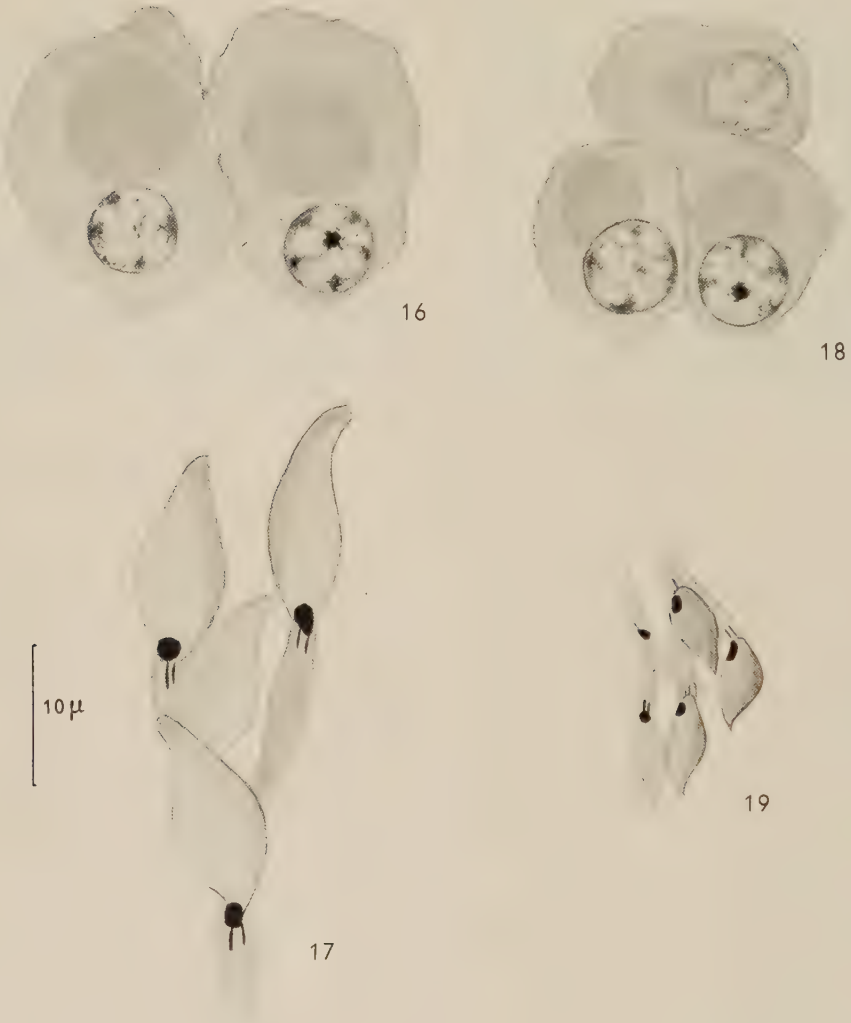
Figs. 14 & 15. Confused stage in normal spermatocytes (Fig. 14) and large spermatocyte (Fig. 15). Volumes of cytoplasm, nucleus, and nucleolus are approximately four times greater in the large than in normal spermatocyte. Photo. 1,500×. (Feulgen and Fast Green.)

spermatocyte nuclei in different lobes of a pentatomid testis may show a constant variation, the chromosomes at metaphase show no corresponding size differences. Thus in *Arvelius* the metaphase chromosomes of the spermatocytes in the large cell lobes (3rd and 5th) are of the same dimensions as those of the much smaller cells and nuclei of the 4th lobe (Figs. 8, 11). Since, further, these chromosomes seem to show the same intensity of staining with chromatin dyes, the assumption that we are dealing with identical amounts of chromatin in the different sizes of cells appeared to be warranted. Nevertheless, such purely observational conclusions concerning the density of a stain cannot be considered as final, and a more objective determination is highly desirable. This was attempted through photometric measurements of the different sizes of spermatocyte nuclei in various comparable stages of mitosis stained by the Feulgen process.

On the basis of such measurements, which are presented in Table II, it appears that spermatocytes in the "confused stage", though varying in their nuclear volumes from 4 to 8 times, have about the same amount of DNA (the differences found being statistically not significant). The photometric measurements, therefore, seem to confirm very well the cytological data that the size of the spermatocyte nuclei in the different lobes is independent of the chromosomal complement. It is further evident from Table II that spermatids immediately after membrane formation, arising from normal or large-sized primary spermatocytes, contain approximately one-fourth of the



Figs. 16 & 18. Early spermatids of large cell lobe (Fig. 16) and small cell lobe (Fig. 18), showing nuclei before differential growth, but cytoplasm and mitochondrial Nebenkern much more voluminous in large spermatid.
Figs. 17 & 19. Later spermatids of large cell lobe (Fig. 17) and small cell lobe (Fig. 19). These are at a more advanced stage than those of Fig. 20.

amount of DNA that is carried by the primary spermatocytes. This is, of course, in accordance with the fact that each primary spermatocyte gives rise to 4 spermatids and implies that in *Arvelius* no DNA is added during

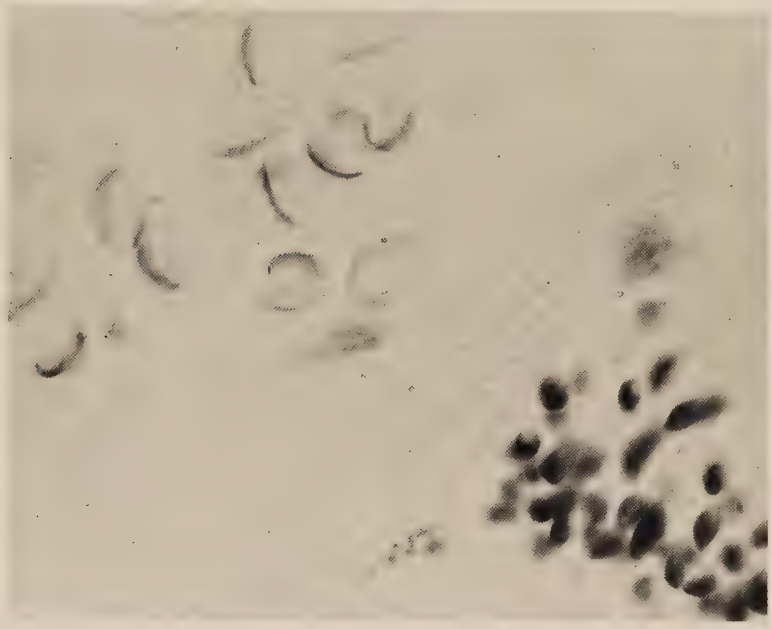


Fig. 20. Spermatids in adjoining large and normal cell lobes. Both sizes of spermatids are beginning to assume ellipsoidal form, but large spermatids are not as dark as small ones. Photo. 1,500×. (Feulgen and Fast Green).

the meiotic divisions. The volume measurements of the different nuclei (see column 4 in Table II) reveal a numerical relationship which may be significant, for it appears that the three different sizes of spermatocytes stand in a multiple relation to each other. Thus, the nuclear volume of the largest spermatocyte is approximately four times as large as the nuclear volume of the normal-sized spermatocyte, while the latter is two times larger than the nucleus of the small-sized spermatocyte.

It is also evident from the table that, in normal cell lobes, a spermatid nucleus (immediately after membrane formation) has $\frac{1}{4}$ the volume of the nuclei in the preceding confused stage, whereas corresponding spermatids in large cell lobes have a nucleus which constitutes only $\frac{1}{16}$ of the volume found in nuclei of the confused stage of their lobe. These numerical relations speak in favor of a great exactness in the growth processes of cells undergoing a normal mitotic cycle, and further investigations in this direction — in *Arvelius* and elsewhere — seem to be called for. As can be seen from Table III and Table IV, there appears to exist a correlation between increase in nuclear volumes and the amounts of contained proteins, indicating a

TABLE II

Comparison of desoxyribose nucleic acid (DNA) (Feulgen) in nuclei of testes of Arvelius.

Lobe of testis	Type of nucleus	Mean diameter of nuclei and plug[1] in microns	Mean nuclear volumes in cubic microns	Number of nuclei measured	Mean per cent of volume of nuclei measured	Mean extinction	Amount of DNA per nucleus in arbitrary units (See footnote 1 page 423)
4th (small cell)	Spermatocyte "confused stage"	7.6 (6.9)	230 ± 32	20	92.8	.125 ±0.01	5.3 ±0.42
2nd (normal cell)	Spermatocyte "confused stage"	9.2 (6.9)	408 ± 43	14	71.7	.109 ±0.01	5.6 ±0.53
5th and 3rd (large cell)	Spermatocyte "confused stage"	14.5 (6.9)	1 596 ± 108	14	32.1	.052 ±0.002	6.0 ±0.24
1st and 6th (normal cell)	Spermatid (shortly after membrane formation)	5.8 (5.8)	102 ± 4	15	100	.048 ±0.004	1.3 ±0.078
3rd and 5th (large cell)	Spermatid (shortly after membrane formation)	5.7 (5.7)	97 ± 4	15	100	.052 ±0.004	1.3 ±0.073

parallel increase or decrease of volume with increase or decrease in proteins. In Table III a few examples are presented in which this relationship seems to be especially striking. Whether these findings are of general implication for any mitotic cycle or apply just to this special case of *Arvelius* remains to be investigated.

IV. THE NUCLEOLUS

1. *Structural basis*

The nucleolus, to which have been ascribed so many functions during the past 70 years, has once more become the subject of much cellular in-

[1] The values in parenthesis represent the diameter of plug measured.

TABLE III

Comparison of proteins (Millon) in nuclei of testes of Arvelius.

Lobe of testis	Type of nucleus	Mean diameter of nucleus in microns	Mean nuclear volumes in cubic microns	Number of nuclei measured	Mean extinction	Amount of protein per nucleus in arbitrary units
6th and 2nd (normal cell)	Spermatocyte "confused stage"	9.2	408 ± 43	11	.161 ±0.014	6.6 ±0.57
3rd (large cell)	Spermatocyte "confused stage"	14.5	1 596 ± 108	11	.168 ±0.010	26.8 ± 1.58
6th (normal cell)	Spermatid (shortly after membrane formation)	5.7	97 ± 4	11	.156 ± 0.013	1.5 ±0.12
2nd (normal cell)	Spermatid	7.2	195 ± 9	10	.126 ±0.006	2.4 ±0.12
3rd (large cell)	Spermatid	7.7	239 ± 8.4	11	.127 ±0.006	3.0 ±0.15
3rd (large cell)	Spermatid (somewhat later)	8.4	310 ± 15	10	.182 ±0.008	5.6 ±0.24

vestigation. This is due largely to the researches of Caspersson and his coworkers (7, 8, 41, 21, 47, 25) which have stressed the important role that is played by the nucleolus in the physiology of the cell. The findings of these workers indicate that the nucleolus represents the accumulation of ribose nucleotides and proteins which originate through some activity of certain regions in the chromosomes (heterochromatin) and are utilized in the formation of the nucleotides and proteins of the cytoplasm.

In view of the significance of these findings a brief outline of the main steps in the nucleolus formation in *Arvelius* is given here. The heterochromatin in the male meiotic nuclei is represented primarily by the two sex chromosomes. In all of the testicular lobes a small nucleolus is present already in the interphase. It was not possible to determine its exact morphological relation to the chromosomes prior to the pachytene stage, for, as in nearly all other pentatomids, these early prophase stages occur in small nuclei whose massed chromosomal threads make a detailed analysis extremely difficult. At synapsis or synezesis the nucleolus often lies in a clear space shared by the heteropycnotic sex chromosomes, but this is not always the

TABLE IV

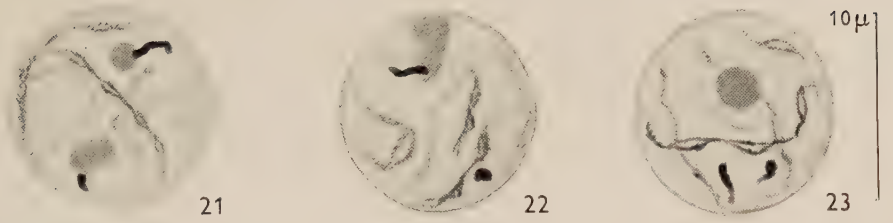
Relationship of proteins in nuclei to nuclear volumes in cells of testes of Arvelius.

Type of nucleus	Mean nuclear volume in cubic microns	Amount of protein per nucleus in arbitrary units	Ratio of nuclear volumes	Ratio of proteins
Spermatocyte of large cell lobe "confused stage"	1,596 ± 108	26.8 ± 1.58	3.9 : 1	4.0 : 1
Spermatocyte of normal cell lobe "confused stage"	408 ± 43	6.6 ±0.57		
Spermatocyte of large cell lobe "confused stage"	1,596 ± 108	26.8 ± 1.58	5.1 : 1	4.8 : 1
Spermatid of large cell lobe	310 + 15	5.6 ±0.24		
Spermatocyte of normal cell lobe "confused stage"	408 ± 43	6.6 ±0.57	4.2 : 1	4.4 : 1
Spermatid of normal cell lobe (shortly after membrane formation)	97 ± 4	1.5 ±0.12		

case. However, during pachytene and diplotene the nucleus becomes greatly enlarged, and observations can then be made with more certainty.

At pachytene the two sex chromosomes in the spermatocytes are heteropycnotic, but instead of the rounded, solidly staining form so typical of many other pentatomids, they are somewhat elongated and betray some indications of coiling. However, by the time that the late diplotene stage is reached both sex chromosomes have become spherical and, that form is retained through the entire confused stage (Figs. 4, 7). Fusion may occur between them, but, as has been reported for several other pentatomids (49, 36, 15), this fusion is a variable process and in many of the spermatocytes is never consummated. Whether joined or not, the sex chromosomes become vacuolated in the latter half of the confused period and finally appear as separate, normal, univalent chromosomes during diakinesis.

As indicated, the morphological relation between the nucleolus and the chromosomes cannot be determined with certainty until the pachytene stage is reached. At that time a small number of nuclei (7 out of 77) showed two nucleoli, but at late diplotene only 2 out of 50 nuclei had this number (Fig. 21). In no case were 3 or more nucleoli encountered. There is thus a tend-



Figs. 21, 22 & 23. Early diplotene stage in large spermatocytes, showing both sex chromosomes, each associated with a nucleolus (Fig. 21); only one sex chromosome associated with the single nucleolus (Fig. 22); neither sex chromosome associated with nucleolus (Fig. 23).

ency to fuse, though it is possible that many nuclei never contain more than one nucleolus from the beginning. The nucleolus increases rapidly in size after synapsis and, like the nucleus itself, attains its greatest dimensions in the confused stage.

Although the earlier stages are difficult to analyze, at early diplotene the two sex chromosomes seem to represent the only heteropycnotic chromosomes in the nucleus. In the majority of cells there is some association between the rapidly growing nucleolus and the sex chromosomes at this time (Figs. 3, 6). But this association is subject to some irregularity, as has been noted for other pentatomids by previous workers (49, 15). Both X and Y, joined or separate, may be in contact with the nucleolus (Fig. 21); only one of them may touch the nucleolus, with the other some distance away (Fig. 22); or, less commonly, neither may be touching or even close to it (Fig. 23). These conditions are shown in the following Table V.

From the data presented it is evident that, in the cases studied, there is an association of the sex chromosomes with the nucleolus during the confused and diplotene stages in at least 70 per cent of the cells. There is also an indication that the association is somewhat higher in the large-sized spermatocytes. Thus during the diplotene stage such association obtains in 84 per cent of these as compared with 72 per cent of the small cells. Whether this greater frequency in the association with the heterochromatin of the sex chromosomes is of importance for the elaboration of a larger nucleolus in these cells — in accordance with Caspersson's theory on the importance of the nucleolus-associated chromatin for growth — cannot be decided in this instance. In any case it appears that most nucleoli are connected with one or both heteropycnotic sex chromosomes during growth, and that even in the late confused stage the great majority of nuclei still show the nucleolus in such contact. It is obvious therefore that some kind of rapport must exist

TABLE V

Association of the sex chromosomes with the nucleolus in spermatocytes of different sizes in Arvelius.

Stage of meiosis	Lobe	X and Y fused or separate, both touching nucleous per cent	X and Y separate, one touching nucleolus per cent	Neither X nor Y touching nucleolus per cent
Confused stage 132 cells	4th (small cell)	32	40	28
Confused stage 75 cells	5th (large cell)	35	49	16
Diplotene stage 106 cells	4th (small cell)	15	57	28
Diplotene stage 68 cells	5th (large cell)	25	57	18

between the sex chromosomes and the nucleolus, for the number of cases that show association between them is much too great to be attributable to chance. On the other hand, it is equally clear that the nucleolus may attain maximum size even though it is in contact with only one sex chromosome for at least part of the time and, in a few cases, with neither — unless there be a connection that is cytologically invisible. However, in these last-named instances where no connection with either sex chromosome is demonstrable, the nucleolus is usually, if not always, in contact with an autosomal thread. That fact may not be without significance in a consideration of the nucleolar origin, and it should be noted that the conditions described here are by no means confined to *Arvelius* but are encountered in some other pentatomids as well. In view of such findings the relations between the nucleolus and the chromosomes merit a general but brief survey here.

In many species, both animals and plants, the nucleolus always arises in conjunction with a specific region of a certain chromosome. This region is usually heterochromatic and in many cases is further characterized by a secondary constriction. There is a growing conviction that such heterochromatin plays a rather direct role in the formation of the nucleolar material which, according to Caspersson (7) and his co-workers, consists largely of ribose nucleotides and proteins rich in dibasic amino acids.

In a consideration of this question it must first be pointed out that neither all secondary constrictions nor all heterochromatic regions are visibly con-

cerned with the origin of the nucleolus. In most cases only one particular heterochromatic region is so involved, and if the others are equally capable of bringing about the formation of nucleolar material, it is only in conjunction with the former that it usually collects to form the orthodox nucleolus.

Further, in many forms and especially the Hemiptera, heterochromatin is chiefly or solely represented in the sex chromosomes. But the degree of heteropycnosis of these chromosomes may differ greatly in different types of cells, and whereas it may be extreme in the meiotic stages, it may not appear at all in the spermatogonia (36, 15). Nevertheless, nucleoli are produced in all these cells.

Finally, it must not be forgotten that in many forms the heteropycnosis of sex chromosomes is correlated with the sex of the individual. An extreme example of this is represented by certain Hemiptera with compound sex chromosomes where the careful studies of Troedsson (48) have thrown much light on this particular question. Thus in the meiosis of the males of *Gelastocoris* and *Acholla* a nucleolus seems to arise in close contact with both the X and Y chromosomes which are extremely heteropycnotic. This is a relationship which is all the more striking since the X of one species has four and the other five separate components, and all, together with the single Y, are in intimate contact with the growing nucleolus. Further, in the female *Gelastocoris,* where, of course, two compound X chromosomes are present, there is no trace of heteropycnosis during the meiotic prophase. Nevertheless, two nucleoli are formed, a fact which taken by itself would seem to argue that the two X chromosomes are indeed involved in their origin. But, obviously, the formation of the nucleoli can here not be due to any direct action of heteropycnotic chromatin in the ordinary sense, for the sex chromosomes are not heteropycnotic in the female and no heterochromatin is visible during the meiotic prophase.

As a result of these considerations, the conditions in *Arvelius* may, for the present, be outlined as follows: The nucleolus arises at certain regions of the chromosomes, i.e., "nucleolus-organizing" regions to use the term proposed by Thorell (47). Both X and Y chromosomes have such a region, and either or both may function in the formation of the nucleolus. Hence two nucleoli are sometimes encountered in the earlier stages (Fig. 21). But in *Arvelius,* and perhaps in some other pentatomids as well, a nucleolus-organizing region is perhaps also present on one or several autosomes, and, though not as efficient as those of the sex chromosomes, such subsidiary organizers may function under exceptional conditions. This would account for the few cases in which a growing nucleolus obviously has no visible con-

TABLE VI

Comparison of proteins (Millon, Fastgreen) and ribosenucleic acid (Azure) in nucleoli of spermatocytes of Arvelius.

Type of nucleolus	Mean nucleolar volume in cubic microns	Amount of protein (Millon) in arbitrary units	Amount of protein (Fastgreen) in arbitrary units	Amount of ribonucleic acid (Azure) in arbitrary units	Ratio of amounts (arbitrary units) in large and normal nucleoli		
					Protein (Millon) Tyrosine	Protein (Fastgreen) Basic Amino acid	Ribonucleic acid (Azure)
Nucleolus of large cell type nucleus "confused stage"	31	1.26 ±0.08	11.6 ± 1.1	0.92 ±0.090	3.1	2.3 : 1	2.4 : 1
Nucleolus of normal cell type nucleus "confused stage"	6.7	0.426 ±0.04	5.0 ±0.5	0.38 ±0.013			

nection with either of the sex chromosomes. All this does not exclude the possibility that a special type of chromatin is involved in the production of the nucleolar material though, as has just been pointed out, such chromatin is not always cytologically recognizable. But clearly, the terms "heteropycnotic chromatin" or "heterochromatin" if applied to such chromatin are then no longer used in the original cytological sense (chromatin that "condenses" at a different rate than euchromatin). Perhaps this nucleolus-forming chromatin had therefore better receive a distinct name such as "plasmochromatin".[1]

Finally, whatever the chemical role played by the "plasmochromatin" may be, it is clear that the nucleolar material is not collected at every place in the nucleus where this type of chromatin occurs but only at very specific localities — usually not more than one or two in number. Certain, but not all, "secondary constrictions" visibly represent such localities (especially in plants), but the nucleolus-organizing region is not always thus recognizable. At any rate, the nucleolus-organizing regions play a unique role in the origin of the nucleolus as it finally appears in the cell, and it is probable that

[1] It is unfortunate that the term "plasmosome", so strongly sponsored by Wilson in his book and earlier, has not replaced "nucleolus" — if only for the practical reason that two confusingly similar names are now applied to such very different cell structures as the nucleus and the nucleolus.

TABLE VII

Relationship of proteins (Millon) in nuclei and nucleoli to nuclear and nucleolar volumes.

Type of nucleus	Mean nuclear volume in cubic microns	Mean nucleolar volume in cubic microns	Amount of protein per nucleus in arbitrary units	Amount of protein per nucleolus in arbitrary units	Ratios between nuclei and nucleoli of	
					volumes	proteins
Large cell type, "confused stage"	1,596 ± 108	31	26.8 ± 1.58	1.26 ±0.08	51 : 1	21 : 1
Normal cell type "confused stage"	408 ± 43	6.7	6.6 ±0.57	.426 ±0.04	61 : 1	15.4 : 1

they have a distinct and special structure. In short, if the plasmochromatin is responsible for the production of the nucleolar material, the nucleolus-organizing region probably has the role of gathering or collecting it.

2. *Photometric measurements of nucleolus*

Ever since the ultraviolet studies of nucleoli by Caspersson and his school, evidence has been accumulated showing that the nucleoli of different tissues and cells contain considerable amounts of ribosenucleic acid (4, 21, 47, 32, 25) and that the main bulk of the nucleolar material is formed by a very basic protein containing diamino acids (7).

The question arose whether the striking differences in the sizes of nucleoli in *Arvelius* spermatocytes (see Tables VI, VII) of the small cell, normal cell and large cell lobes are associated with differences in the amounts of nucleolar material, or whether like the DNA a uniform nucleolar material is diluted to varying degrees. For this purpose the large nucleoli were compared with the small nucleoli in spermatocytes of corresponding meiotic stages as to their protein content and as to the amounts of ribosenucleic acid carried by them. As Table VI shows, the large nucleoli obviously contain by far the greatest quantities of the three constituents measured, carrying from 2 to 3 times the amounts that are found in the normal nucleoli.

Another aspect which arose from these studies of the nucleolus is that (as can be seen from Table VII) the nucleolar volume in relation to the nuclear volume is approximately the same in the large spermatocytes and in the small spermatocytes. The difference between $\frac{1}{50}$ and $\frac{1}{60}$ of the total volume is probably not significant and perhaps due to errors arising from the computation of the nucleolar volume from non-spherical nucleoli. On the basis of the data

presented in this table it is further evident that the concentration of protein in the nucleoli is considerably higher than the concentration of the proteins in the nuclei (Table VII). This finding is in agreement with data presented by Hydén (21, p. 112) for the nucleoli of the nerve cells of *Lophius piscatorius*, showing that the concentration of protein in the nucleoli as measured by the ultraviolet absorption at 2,800 Å is considerably higher than the concentration in the nucleus. Hydén also showed in accordance with the results presented here that the quantities of ribosenucleic acid (as determined by the ultraviolet absorption at 2,600 Å) and of proteins in larger nucleoli of nerve cells were considerably greater than those of smaller nucleoli.[1]

V. THE CYTOPLASM

1. *Structural basis*

The size differences observed in the spermatocytes of the different lobes of the testis are not restricted to the nuclei and nucleoli but extend to the cytoplasm as well (Figs. 4, 7). However, it is difficult to obtain accurate measurements of cytoplasmic volumes, especially in the small and normal-sized cells because their outlines are irregular and variable. Nevertheless, it is safe to state that in the middle of the confused stage the volume of normal-sized cells is approximately twice that of the small cells, while the large cells are about 8 times as large (Table I). The relationship of the cellular volumes and cytoplasmic volumes is therefore approximately the same as the nuclear relationship in the different sizes of spermatocytes, namely, that each of the values is a member of a geometrical series. These comparative dimensions, in so far as the small and large cells are concerned, were already tentatively determined by Bowen (3) whose conclusions were confirmed by Schrader (39), and we now found again that these values are approximately correct.

2. *Photometric measurements of cytoplasm*

The striking differences in the amount of cytoplasm in the different sizes of spermatocytes led to the determination of proteins and ribosenucleic acid in a manner similar to that used for the nucleolus. In the following table (Table VIII) extinctions are presented which were obtained after staining with Millon, Fast Green and Azure.

[1] A comparison of the Fastgreen-Millon ratio of the cytoplasm with that of the nucleolus seems to indicate that the latter contains a protein richer in diamino acids, in agreement with the conclusion of Caspersson and Hydén.

TABLE VIII

Comparison of proteins (Millon, Fastgreen) and ribosenucleic acid (Azure) in the cytoplasm of spermatocytes of Arvelius.

Type of Cytoplasm	Mean cytoplasmic volume in cubic microns	Mean amount (arbitrary units) of			Ratio of amounts (arbitrary units) in the cytoplasm of large and normal sized cells of		
		Protein (Millon)	Protein (Fastgreen)	Ribosenucleic Acid (Azure)	Protein (Millon)	Protein (Fastgreen)	Ribosenucleic Acid (Azure)
Cytoplasm of large cell type "confused stage"	8,278 ± 801	124 ± 12.4	790 ± 64	93.5 ± 6.0	3.75 : 1	3.49 : 1	2.82 : 1
Cytoplasm of normal cell type "confused stage"	2,545 ± 245	33 ± 5.8	226 ± 19.2	30.2 ± 3.2			

As can be seen from Table VIII, the cytoplasm of the large-sized spermatocytes contains considerably more protein (as tested by the Millon reaction and Fast Green staining) and more RNA (as tested by Azure stain) than the cytoplasm of the normal-sized ones in the same meiotic stage. It is evident from these results that within the cytoplasm the ribosenucleic acid increases proportionally with the changes in volume and protein content. This is in good agreement with the behavior of the ribosenucleic acid in the nucleolus, but in contrast to the behavior of the DNA in the nucleus which stays unchanged while the protein increases.

VI. DISCUSSION OF THE RESULTS

1. *The nucleus*

The findings concerning the nucleus may be summarized as follows:

a) The volumes of the nuclei of the large cells are about 4 times greater than the nuclei of the normal and about 8 times larger than the small cells.

b) A direct relationship appears to exist between nuclear volumes and relative amounts of proteins (as tested by the Millon reaction). The larger the nucleus the greater the total quantity of proteins and vice versa.

c) In striking contrast to this correlation between proteins and nuclear sizes is the constancy of the DNA. All the nuclei, varying in the different lobes from about 200 to 1,600 cubicmicrons, showed approximately the same amount of DNA (as tested by the Feulgen reaction).

The occurrence of nuclear sizes of the spermatocytes of *Arvelius* in a geometrical series (see Table I) supports the hypothesis of Jacobj of a "rhythm in cell growth" rather well (22, 23). On the basis of many measurements of nuclear volumes on a large series of tissues of mammals Jacobj presented data showing a remarkable relationship of 1: 2: 4: 8 of nuclear volumes in nuclei of different tissues. We found that in *Arvelius* also the nuclear volumes of the primary spermatocytes in the different lobes showed such a relationship of 1: 2, or 1: 4, or 1: 8, furthermore, the spermatids shortly after membrane formation were exactly one fourth the volume of spermatocyte nuclei (in the confused stage) of the small cells, and one-sixteenth of corresponding nuclei in the large cells. While these findings in *Arvelius* conform to Jacobj's hypothesis, Hertwig's statements (18, 19) that the multiple growth of cells is caused by a multiplication of the mass of chromosomes or is necessarily correlated with an increase in chromosomal number does not apply to the cells of the testis of *Arvelius*. From the cytological analysis of Montgomery (30), Bowen (3) and Schrader (39) it is evident that the different sizes of nuclei contain the same number of chromosomes, which, moreover, compared exactly in size so far as could be determined cytologically (Figs. 8–13). That Hertwig's interpretation is not of general applicability is also shown by Höppner's study (20) which described a typical geometrical series for the nuclear volumes of Fucus in which all nuclei carry the same haploid number of chromosomes. The findings of Lauber (26) on angiosperms seem also to speak against a strict relationship between chromosomal number and nuclear or cellular volumes. According to Lauber the increase in nuclear volumes of tetraploid over diploid cells is often considerably greater than would be expected from the chromosomal number; she also found that some diploid nuclei in the plant *Curcubita maxima* have a nuclear volume which is nearly as large as the nuclear volume of the tetraploid nuclei. Unfortunately, these data are not too conclusive since the plant is rather unfavorable from a cytological point of view; the chromosomes are very small and the nuclei are irregular in shape, which would make it very difficult to determine with precision the polyploidy and the nuclear volumes. The problem as to the plausible cause of the rhythmic growth and its correlation to increase of nuclear and cellular volumes will be discussed later in connection with the question of protein synthesis.

In full accordance with the uniform number and size of chromosomes in the different lobes of the testis of *Arvelius* is the DNA content which remains unchanged in the spermatocytes of different sizes as well as in spermatids of different sizes. In this connection it may be worth while to mention that even

in cases of known polyploidy — such as for instance in the mouse liver (22) — where multiples of DNA content have been reported (35, 45) the increase in nuclear volume could hardly be accounted for by the changes in the DNA content alone, but must involve additions of proteins as well. The relationship of proteins to nuclear volumes in polyploidy and polytene nuclei is under study. Our interpretation, that the amounts of proteins are of importance in explaining increases in cell and nuclear sizes, is based on data brought forward recently showing that in comparison with the protein the DNA seems to account for only a small fraction of the total nuclear material. In contrast to the chemical analysis of isolated animal nuclei by Mirsky and Ris (29) in which ratios of protein to DNA such as 1.5: 1 were obtained, or the results of Stedman and Stedman (44) who reported ratios of 2.6: 1, the photometric analyses of animal nuclei of whole cells showed considerably higher values for proteins in nuclei. Caspersson and his school (6, 7) — using the natural absorption of nucleic acids in the ultraviolet at 2,600 and 2,800 Å — obtained in nuclei ratios of protein to DNA varying from 10: 1 to 20: 1, depending on the type of cell investigated. Pollister and Leuchtenberger (32) — using light absorption after the Feulgen reaction for DNA and the Millon reaction for protein — found a good agreement with the ratios of Caspersson, namely, from 8: 1 to 17: 1 in different cells. The discrepancy between the chemical and photometric analysis is apparently due to a loss of protein during the isolation procedures in the former (13, 32). Granting the possibility that in the photometric measurements the values for the proteins are perhaps somewhat too high — an error possibly arising from different distributions of DNA and proteins in the cells analyzed, — the fact remains that nuclei contain considerably higher amounts of proteins than DNA. Confirmation of such a conclusion by chemical techniques is afforded by the recent analysis of rat liver cells by Price *et al.* (34). These authors report a ratio of 10: 1 between protein and DNA in the nuclei which is not too far removed from the ratios obtained in photometric analysis (32).

The assumption therefore seems justified that changes in nuclear sizes in a tissue are due mainly to alterations in protein and water content and not due to different amounts of DNA. This concept is also in agreement with the findings that in the early pycnosis of tumor cells of mice, where a marked reduction of the nuclear volume occurs, a considerable drop in the protein content of the nuclei is found, whereas the DNA remains unchanged in quantity (28).

In connection with the relation of protein to cellular size it seems of importance to discuss "growth by hydration" which is so often mentioned in

the cytological literature. Growth of cells which cannot be explained by an increase in chromosomal number or chromosomal mass is frequently interpreted as a hydration process (16). Independent of the studies reported here, where a direct correlation between amount of proteins and cellular growth could be demonstrated, the occurrence of a rhythmic growth of cells in Fucus in which the chromosomes stayed unchanged as to number and mass (20) strongly contradicts the concept of "growth by hydration". Indeed, it seems rather unlikely that water should be taken up by nuclei and cells in a fashion so rhythmical that exact multiples of the volumes of nuclei and of cells result. Furthermore, a volumetric increase of a cell to 4 times its original volume, solely as the result of an imbibition of water, might be expected to lead to alterations in the osmotic conditions which are hardly possible in the physiology of a normal cell. This, of course, does not imply that water uptake does not play a role during growth, but the point to be stressed here is that the growth of cells cannot simply be explained by a sudden hydration. The center of cellular growth is the formation of new proteins.

The importance of protein synthesis for the "rhythmic growth of cells" seems to gain further support if one considers the protein volumes in different sizes of nuclei of *Arvelius* in relation to the volumes of these nuclei. (See Table IV.) The amazing parallelism between amounts of proteins and nuclear volumes — both being, for instance, 4: 1 for two different size classes of spermatocytes, or 4: 1 for a spermatocyte and its spermatid — seems to be more than a strange coincidence (Table IV). One cannot help but weigh the possibility that perhaps under certain circumstances, where a regular and orderly growth is of greatest importance (such as is the case in mitosis and meiosis) protein synthesis might itself be a rhythmic process. The production of 4 equally sized spermatids from each primary spermatocyte and the regular and equal distribution of the spermatocyte material (namely, one-fourth of the DNA and one-fourth of the protein) which that involves shows that in the development of the spermatocyte there must be an exact anticipatory regulation of the growth process.

The question of the interrelation of protein synthesis and DNA synthesis arises in connection with the different behavior of the DNA and proteins in the different spermatocytes, the former staying practically unchanged in quantity while the latter is subject to changes, as has just been discussed. The inference seems justified that DNA synthesis and protein synthesis in the same cell are independent processes in the sense that protein synthesis may occur without simultaneous DNA synthesis. But the question now arises whether such a protein synthesis involves all or only certain of the

cellular proteins. Caspersson (7) has suggested that two processes may be distinguished in relation to cellular protein synthesis. The first process involves the reproduction of the "genic proteins" and the second is concerned with the formation of the main bulk of the "cytoplasmic proteins". In *Arvelius* two different types of synthesis are clearly indicated — the synthesis of what might better be called "chromosomal proteins" and the synthesis of "extrachromosomal proteins" — and our analysis strongly intimates that the latter can take place independently of the former. For it must be remembered that the increase of proteins in the cytoplasm, the nucleolus and the karyoplasm of the nucleus of the large cells, as compared with the normal and small cells, is not accompanied by an increase in chromosome number, chromosome size or DNA. Any increase of protein in the chromosomes would of necessity cause an enlargement of chromosomal volume accompanied by a corresponding dilution of the DNA in such chromosomes, and the cytological evidence shows that such changes do not occur (Figs. 8–12). A separation of the synthesis of "chromosomal" proteins and the synthesis of "extrachromosomal" proteins in the same cell intimates in turn that probably two different enzyme systems are involved in the two processes. Any explanation based on the assumption that a single enzyme system is responsible for both processes would meet with great difficulty in accounting for the independence in the synthesis of these two types of proteins. Finally, the constancy of the amounts of DNA and "chromosomal" proteins argues that the two are closely linked as to their synthesis.

The concept of different enzyme systems for such processes as chromosomal division (which involves a synthesis of desoxynucleo-proteins) and, on the other hand, growth, is also inherent in the work of Lettré and Lettré (27) on mitotic poisons. Thus the poisoning action of metallic organic compounds on the division of chromosomes can be neutralized by the addition of compounds (cystein, thiophenol) contanining -SH groups, while a poison like colchicine, which does not seem to act on the chromosomes themselves but on the extrachromosomal constituents like the spindle, is not neutralized by such compounds. Since it has been known for some time that a large number of enzymes requires the presence of -SH groups in their protein moiety for activation, the possibility that such an enzyme system is involved in the synthesis of "chromosomal proteins" and DNA should be considered.

The nature of the evidence for a separation of the protein synthesis in *Arvelius* suggests that other natural instances may show a reciprocal condition in which DNA and "chromosomal" proteins are synthesized, while the "extrachromosomal" proteins remain constant in amount. Such a condition

seems to obtain in the case of Sorghum (10) where increased amounts of chromosomal material are carried in cells which seem to undergo no corresponding increase in "extrachromosomal" proteins. A cytochemical analysis of such cases may, indeed, complete the demonstration that the two different types of protein synthesis occurring in the cell may proceed independently of each other. The separation of the two processes in *Arvelius*, namely, growth of cells — or more specifically protein synthesis — on the one hand and the synthesis of DNA on the other, seems to be in very good agreement with the particularly interesting experiments of Bodenstein and Kondritzer (2). These workers showed that, when amphibian embryos were exposed to nitrogen mustard, the synthesis of DNA and cell division were inhibited, while the increase of the ribosenucleic acid as well as growth and differentiation of the cells were not affected. As a result, cells so treated reached giant proportions. Although Bodenstein and Kondritzer made no analysis of the various cells for proteins, their findings on giant cells with large nuclei and large nucleoli, as well as the increase in RNA, strongly suggest an unimpared protein synthesis. The question arises whether the occurrence of giant cells in the mustard-treated material, as well as in *Arvelius*, might not be explained on some such basis as the following: In the normal mitotic cycle a cell builds up exact quantities of DNA and proteins, as well as the other chemical constituents of cells (such, as for instance, RNA and the carbohydrates, which were not investigated here) for the ensuing daughter cells. The blocking of the DNA synthesis by the nitrogen mustard prevents mitosis and division but does not interfere with the synthesis of the other constituents. This results in single cells with an accumulation of proteins which actually represent the complement for a number of cells. While no nuclear size measurements or chromosome counts are reported in Bodenstein's work, the nearly exact multiples of nuclear volumes (namely, 200, 400 and 1,600 cubicmicrons) which we found in *Arvelius* and their close correlation with the amounts of proteins carried in such nuclei seem to favor such a hypothesis.

Certain conditions in the *Arvelius* testis suggest a decrease in mitotic activity such as is reported by Bodenstein. Thus the number of cells prior to the synaptic stage is very much smaller in the large cell lobes than in the other lobes; rough counts put this number at approximately 350 to 500 in a large cell lobe (the 5th) and at upward of 15,000 in the lobe with the smallest cells (the 4th). This may bespeak an impeded mitotic activity in the former, but unfortunately too little is known of the embryological development of the pentatomid testis to allow a final decision. Thus it is at least conceivable

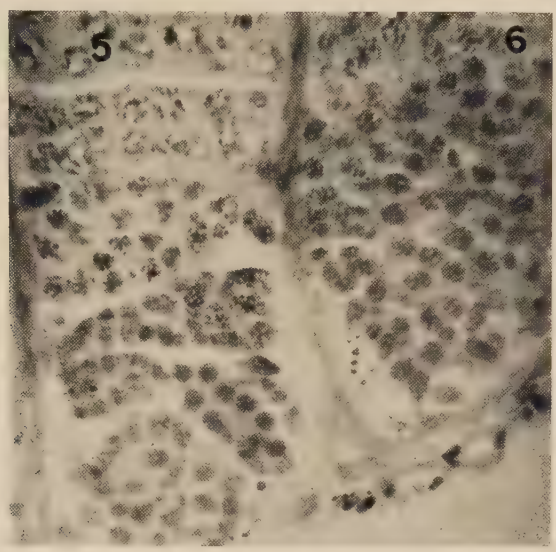


Fig. 24. Terminal regions of adjoining large cell (5th) and normal cell (6th) lobes. Lowest cells are spermatogonia which are similar in size in the two lobes, but large cell lobe (on left) shows differential size increase of cells in leptotene and pachytene stages at upper end of photograph. Photo. 500 ×. (Feulgen and Fast Green).

that the lobes with the higher number of cells contained more cells to start with, although Seidel's work (42) on the embryology of the distantly related hemipteran Pyrrhocoris uncovered no such differences. Certainly if a slowing of the mitotic activity occurs, it does not act so as to increase the size of any of the nuclei and cells prior to the synaptic period in meiosis (Figs. 2, 5, 24). The reaction is, therefore, different in meiotic and premeiotic cells. Further, if the rate of division is lowered in the large cell lobes, no effect is thereby exerted on the number of meiotic divisions; there are two in all the lobes — neither more or less (Figs. 8–13). But for the present, Bodenstein's findings furnish the best working hypothesis in the analysis of size differences in the *Arvelius* testis even though the conditions there offer some complications which call for further embryological data.

A separation of the protein synthesis from the DNA synthesis has also been demonstrated in nondividing cells, such as liver cells. In a number of nutritional experiments (11, 24) chemical analyses of liver showed that, after starvation or low protein diet, there was a considerable drop of the RNA and a marked decrease in the cellular volume of the liver, while the DNA remained nearly unaffected. In a recent extensive cytological study of the

protein metabolism of the liver in rats Lagerstedt (25) although in general agreement with the results of Davidson and Waymouth and Kosterlitz, stresses the disappearance of the nucleolus-associated chromatin (as judged by the Feulgen stain) during starvation and low protein diet and its increase under high protein diet, thus implying that the DNA might be at least to some degree reduced when protein synthesis is impaired. It would be of interest to investigate whether photometric measurements show an actual loss of DNA from such nuclei or perhaps only a different distribution of the DNA within the nucleus.

The stability of the DNA during growth and protein synthesis of cells in *Arvelius* is also in accordance with the results of the isotope work of Brues (5) and other workers which show that the DNA undergoes very little turnover as compared with the RNA and proteins.

2. *The nucleolus*

The various results concerning the nucleolus may be briefly summarized:

a) In the confused stage of the spermatocytes the nucleoli of the large cells contain about 2 3 times more proteins and ribosenucleic acid than do the nucleoli of the small cells.

b) The concentration of proteins in these spermatocyte nucleoli is about 2.5 to 5 times greater than is the concentration of proteins in the nuclei of the same spermatocytes.

According to Caspersson and his school, the nucleus is the center of protein synthesis, and the rapidity of the growth of a cell can be judged by the size and composition of the nucleolar apparatus (47, 7). The findings in *Arvelius* on the nucleolus and its relation to the growth of the cells are in full support of this idea. The larger the nucleolus and the greater its contained quantity of proteins (as judged by tyrosine and by the diamino acids) and ribosenucleic acid, the larger the spermatocytes and the greater the amounts of proteins in nuclei and cytoplasm. Whether there is a *causal* relationship between changes in nucleolar apparatus and growth of the cells — such as is suggested by Caspersson — cannot be judged in *Arvelius*, since here the increase in nucleolar size (from synapsis to confused stage) runs parallel with an increase in nuclear and cellular size. We also cannot say whether in *Arvelius* the higher concentration of proteins in the nucleolus as compared with the lower concentration of proteins in the nucleus is an expression of a gradient concentration of proteins from the nucleolus towards the nuclear membrane. The concentration of the proteins in the nucleus as presented

here is based on measurements of proteins on a large area of the nucleus — and thus presents an overall picture — while Hydén (21) studied different points in the nuclei of the nerve cells of *Lophius piscatorius* and thus established the gradient of the protein concentration. Otherwise the cytochemical results in the nucleoli and nuclei in *Arvelius* are in full accord with the results of Hydén in nuclei and nucleoli of different sizes in the nerve cells of *Lophius*.

In contrast to the correlation between nucleolar size, nucleolar substances and cell size (or more specifically protein synthesis) is the relationship of DNA to nucleolar size in *Arvelius*. The increase of the nucleoli to nearly 5 times their volume, with a corresponding increase in ribosenucleic acid and proteins, is by no means reflected in the quantity of the DNA. The latter, as here pointed out, remains approximately the same. These results are in contradiction to the statement of Heidenhain (17) that the size of a nucleolus is directly dependent on the amount of chromatin in the nucleus. The results in *Arvelius* also do not seem to support the view that the increase in nucleolar volume during protein synthesis is necessarily accompanied by a larger amount of DNA in the form of nucleolus-associated chromatin. If there is such an increase in the quantity of the DNA, then it must be in an order of magnitude which cannot be detected by the Feulgen reaction and by the type of photometric measurement used in this study. The conditions in *Arvelius* seem to stress an intimate relationship between nucleus, nucleolus and cytoplasm from the point of view of protein synthesis, whereas DNA synthesis appears to be an independent process and its immediate connection with the others is not clear.

3. *The cytoplasm*

The various results concerning the cytoplasm may be briefly summarized:

In the confused stage of the spermatocytes the cytoplasmic volume of the large-sized cells is over three times larger and contains over three times more protein and ribosenucleic acid than the cytoplasm of the normal-sized cells.

These data indicate that as one goes from the normal to the large-sized cells, not only is an increase in the nuclear and nucleolar volume encountered, but also a proportionate addition to the cytoplasm. As in the nucleus and nucleolus this change in the volume of the cytoplasm is accompanied by a rise in the protein content, and therefore the enlargement of the cytoplasmic dimensions involves a growth in the true sense of the word. The corresponding increase in the ribosenucleic acid with the growth of the cytoplasm and of the nucleolus suggests a possible linkage of these two processes,

an assumption which has already been expressed by Muller (31), Spiegelman and Kamen (43), and Caspersson (7). These authors suggest that the ribosenucleic acid is essential for the synthesis of the cytoplasmic proteins, probably acting as an energy donator.

The increase of proteins and ribosenucleic acids in the cytoplasm of the large-sized spermatocytes as compared with the small and normal-sized ones — established by the cytochemical analysis in this study — is supported by the cytological findings (Figs. 2–13). Bowen, who was primarily and predominantly interested in the structural elements carried in the cytoplasm, concluded that the Golgi material, the chromatoid bodies and the mitochondria are all present in greatly increased quantities in the large cells. This conclusion can be confirmed without difficulty, for the Golgi material associated with the acrosome and the massed mitochondria which come to form the Nebenkern in the spermatid (Figs. 16, 18) are quite obviously several times more voluminous in the large than in the normal-sized and small cells.

It is perhaps of some interest to refer briefly to the condition of the spindles in the various sizes of cells. So far as *Arvelius* is concerned, it is likely that most of the spindle substance is derived from the nucleus itself, but this is not true of all organisms since in certain forms the cytoplasm also participates in spindle formation. A cytoplasmic origin is even more likely for the astral fibers which extend all through the cytoplasm at metaphase.

The spindles themselves are greatly enlarged in the large cells (3, 39) and their staining reactions strongly indicate that this increase represents an increase of spindle substance and not a dilution. More exact data cannot be given for the present, but even as it stands this finding is of interest in the old question of the origin of the spindle. Thus it has occasionally been claimed that the spindle is, at least in part, produced by the chromosomes themselves — let us say as a sort of secretion (see discussion by Wilson, (50)). The constancy of chromosome size and, even more, the uniformity of the amount of DNA in the cells which produce spindles of different dimensions, furnish a strong argument against any such direct derivation of the spindle substance from the chromosomes, but make it more probable that the extrachromosomal protein in the nucleus contributes to the formation of the spindle.

VII. SUMMARY

1. The functional interrelations of nucleus, nucleolus and cytoplasm have been studied cytochemically in material which is peculiarly favorable for

such an analysis. The different lobes of the testis of *Arvelius albopunctatus* — a hemipteran insect — carry spermatocyte and spermatid cells which differ regularly in size but are otherwise normal in their meiotic processes. Thus two of the lobes are characterized by very large cells, three by normal-sized cells, and one by small cells. In these the protein, DNA and RNA content (in arbitrary units) of the nucleus, nucleolus and cytoplasm were correlated with the volume of these cell structures with the following results:

2. The Nucleus: The nuclear volumes of the three sizes of spermatocytes were 200, 400 and 1,600 cubic microns, constituting members of a geometrical series. The increase of total proteins in these various nuclei corresponds to the increase in their volumes, while the DNA was approximately the same in all of them. The nuclear volumes of the spermatids (immediately after nuclear membrane formation) is exactly one-fourth and one-sixteenth of spermatocyte nuclei in the confused stage, depending on whether they derived from a normal or large spermatocyte. Similarly, spermatid nuclei of the normal cell lobe carry one-fourth of the proteins and DNA that are present in spermatocyte nuclei prior to the two meiotic divisions in that lobe.

3. The Nucleolus: The volume of the nucleolus in the large spermatocytes is about four times that of the nucleolus in a normal spermatocyte. The total protein and RNA of such a large nucleolus is proportionally increased whereas the amount of DNA of the nucleus remains unaltered.

4. The Cytoplasm: The ratios of the volumes of the cytoplasm in the three different sizes of spermatocytes have approximately the same order of magnitude as the ratios of nuclear volumes. The increase in the amount of cytoplasm in the normal and large cells over the small cells is accompanied by a rise of proteins and RNA in the cytoplasm.

5. These cytochemical data as well as the cytological analysis lead to the conclusion that in *Arvelius* the increase in the volumes of the nucleus, nucleolus and cytoplasm, as seen in the normal and large cells as compared with the small cells, represents a true growth. In contrast to these changes in the amounts of proteins and RNA is the quantity of DNA which remains unaltered in the different sizes of nuclei and cells. The findings imply that protein and DNA synthesis can proceed independently of each other, whereas the synthesis of RNA and proteins are closely linked.

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CHROMATOGRAPHIC STUDIES OF AMINO ACIDS IN THE EGGS AND EMBRYOS OF VARIOUS SPECIES[1]

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Experiments involving the use of paper partition chromatography have been undertaken to study embryonic development in its possible relation to changes in the amino acid composition of protein in developing tissues. The present investigation is divided into three parts:

I. A study of the protein and non-protein nitrogen fractions of the various developmental stages of the frog egg.

II. A study of the amino acid composition of isolated morphologically defined embryonic fragments and organs of successive developmental stages of several amphibian species.

III. A survey of the amino acid composition of various vertebrate and invertebrate eggs.

These investigations were undertaken in an attempt to test one aspect of the general idea that embryonic differentiation is the morphological expression of an underlying chemo-differentiation of the cellular constituents involved, particularly of the proteins. It was thought that the relatively simple and yet highly sensitive and discriminating technique of paper partition chromatography might reveal characteristic differences in the chemical composition of the successive stages of the developing egg and its different portions, thus furnishing biochemical data on processes which, so far, have been described and interpreted almost exclusively in the terms of classical embryology. Amphibian eggs were chosen as the principal material because they represent one of the best investigated subjects of experimental embryology and lend themselves readily to the surgical and chemical manipulations here employed.

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METHODS

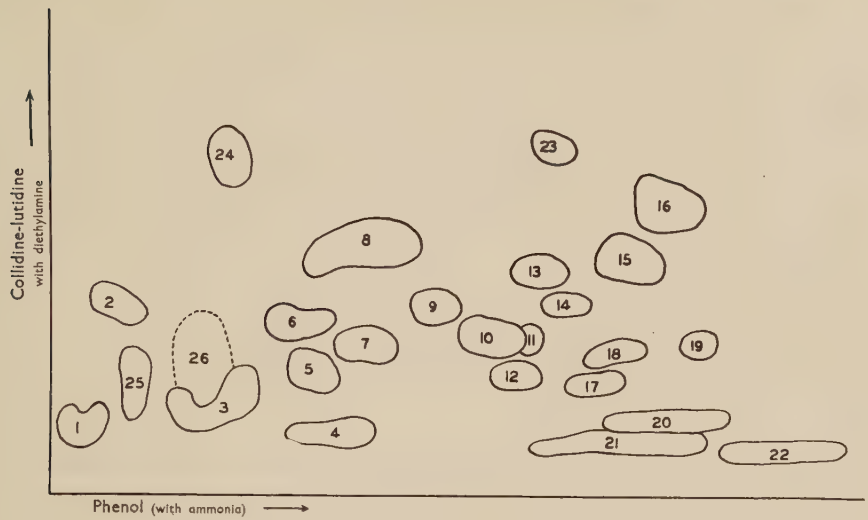
Tissues were prepared and preserved for analysis by freezing in an ordinary refrigerator. When studied they were thawed out rapidly and hydrolyzed with a threefold excess of 6N HCl solution by volume, or with 20 mg $Ba(OH)_2$ per 200 microliters solution for 18 hours at 100°C. Control analyses with freshly prepared material were made whenever possible and gave results identical with those from frozen tissues. In the case of the alkaline hydrolysis, the solution was neutralized with CO_2, and $BaCO_3$ removed by centrifugation.

Tissues which were examined for non-protein nitrogen components were triturated and extracted three times with 75 per cent ethyl alcohol at room temperature. The alcoholic solution was evaporated in vacuo and the residue extracted first with cold water and then with ethyl ether. The aqueous fraction was again extracted with ether (three times) and then chromatographed in its original state and also after oxidation on the paper with hydrogen peroxide. The hydrochloric acid and barium hydroxide hydrolysates of the protein residues were treated in the same way. Chromatograms were prepared according to the technique of Dent (2). The amino-acids were identified by their relative positions on the two-dimensional chromatogram and by comparison with the reference map of Dent (2). Determinations were made in duplicate in practically every case.

A semi-quantitative method of determining amino acids essentially similar to the technique of Polson (4) was developed in this laboratory. Aliquot samples of tissue acid hydrolysates were analyzed for nitrogen content by Kjeldahl digestion, nesslerization, and colorometric estimation. Subsequent chromatograms were made equivalent on a total nitrogen basis. It was found with the particular amino acids studied that differences of 1 γ would produce a detectable color intensity change of the ninhydrin developed "spot" in a range of 1–6 γ of amino acid. Under the best conditions the amino acids studied could be estimated with an accuracy of $\pm$ 15 per cent. Each chromatogram was made on the basis of 7 γ total nitrogen content since the concentration of the amino acids studied fell in the required working range. Standards prepared from authentic specimens of amino acids were always run with the experimentals under the same conditions in individual chromatography boxes.

RESULTS

I. Acid and basic hydrolysates of whole frog eggs (*Rana pipiens*) stripped of their gelatinous membranes were first investigated. Analyses were performed on ovarian and uterine eggs and on successive stages up to the larva. The material studied included the two-cell stage, morula, blastula, early, middle, and late gastrula, early neurula, and swimming larvae (8 mm). No significant qualitative differences in amino acid composition were found, the chromatograms of all the stages tested being almost identical



(1) Unknown substance found in egg HCl hydrolysates of the sun fish and brine shrimp. (2) Cysteic acid. (3) Aspartic acid. (4) "Underglutamic Acid". (5) Glutamic acid. (6) Serine. (7) Glycine. (8) Taurine (tentatively). (9) Threonine. (10) Alanine. (11) Hydroxyproline. (12) Glutamine (tentatively). (13) Methionine Sulfone. (14) α-amino-n-butyric. (15) Valine. (16) Leucines. (17) Histidine (tentatively). (18) Unknown substance found in ovarian egg alcohol extract of *R. pipiens*. (19) Proline. (20) Lysine. (21) Arginine. (22) "Fast Arginine". (23) Tyrosine. (24) Glucosamine (tentatively). (25) Unknown substance found in egg HCl hydrolysates of the mollusc, *Crepidula fornicata*. (26) Yellow unidentified spot not reacting with ninhydrin.

and corresponding closely to the composite diagram of Fig. 1. A complete list of the amino acids found is contained in Table II. It appears, from evidence presented later in this paper, that any quantitative change in amino-acid composition during development is at most of a low order as revealed by a comparison of the amino acid composition of the unfertilized ovum with that of the various germ layers of the early gastrula and neurula stages.

II. Supplementing the analyses on whole eggs and embryos, determinations were made on well defined embryonic fragments which were isolated by means of fine glass needles and then subjected to acid hydrolysis. A group of about five identical fragments provided sufficient material for a clear chromatogram. Separate analyses were made of the dorsal and ventral halves of neurulae of Xenopus laevis, and of the ectoderm, endoderm, upper blastopore lip, and ventral marginal zone from gastrulae of Amblystoma punctatum and Xenopus laevis. With the aim of detecting possible differences in the relative amounts of several of the amino acids present in different germ layers, the semi-quantitative method mentioned

TABLE I

Semi-quantitative data on isolated fragments from different germ layers of *Xenopus laevis* neurulae and gastrulae

7 micrograms total nitrogen content per sample	Micrograms amino-acid
Glutamic acid	6—8
Aspartic acid	2—4
Serine	4—6
Glycine	2—3
Alanine	3—5

previously was employed. Such estimations carried out on aspartic acid, glutamic acid, glycine, alanine and serine, showed that their relative concentration from tissue to tissue in the isolated embryonic fragments did not vary significantly (Table I). The following data represent over-all average values obtained from chromatograms run at least in triplicate on each of the various embryonic segments studied: namely, ectoderm, entoderm, and upper blastopore lip of early gastrula, and the dorsal and ventral halves of early neurula of Xenopus laevis.

These particular acids were selected for analysis because they occurred in the highest concentration and in relatively comparable amounts. Additional, merely qualitative assays likewise revealed a striking similarity in the total amino acid composition of the various germ layers (Table II). This similarity applied furthermore to adult tissues such as muscle, heart, liver, lung, brain and spinal cord, the hydrolysates of which were investigated separately.

Preliminary work has been undertaken with the examination of alcoholic extracts of whole ovarian eggs for free amino acids, polypeptides, and related compounds. The concentration of these low molecular weight water-soluble substances appears to be exceedingly small in ovarian frog eggs. A cold alcoholic extract of the contents of four ovaries (40 gm fresh weight) produced barely enough material for three readable chromatograms. Markedly better results were obtained with extractions carried out in a soxhlet continuous extractor for 18 hours with 75 per cent ethyl alcohol. Chromatograms made from material prepared by either the cold alcohol or the soxhlet extraction technique corresponded closely to one another. In contrast to the negative results of Li and Roberts (3), practically the full

TABLE II

Similar amino-acid composition found in the following embryonic material

Ova of		Ova of		Tissues of
Crepidula fornicata	(mollusc)	*Etheostoma b. blenniodes*	(green-sided darter)	Chicken embryos (18–20 hrs.)
Busycon canaliculatum	(conch)	*Lepomis gibbosus*	(common sun fish)	*Rana pipiens* (leopard frog)
Chaetopleura apiculata	(bivalve)	*Beirachus* sp.	(toad fish)	1. successive developmental stages
Venus mercenaria	(clam)	*Fundulus heteroclitus*	(killifish)	2. adult tissues (brain, muscle, liver, heart, duodenum, lung, spinal cord)
Loligo pealeii	(squid)	*Rana pipiens*	(leopard frog)	Germ layers of *Xenopus laevis* and *Amblystoma punctatum*
Asterias forbesi	(starfish)	*Amblystoma punctatum*	(spotted salamander)	1. early gastrula ectoderm, entoderm, upper blastopore lip, ventral marginal zone
Arbacia punctulata	(sea urchin)			2. neurula dorsal half, ventral half
Artemia sp.	(brine shrimp)			
Limulus polyphemus	(horse shoe crab)			

Amino-acids found in all the material indicated above

glutamic acid	leucines	threonine
aspartic acid	cystine (as cysteic acid)	methionine
glycine	tyrosine	proline
alanine	arginine	histidine (?)
valine	lysine	α-amino-n-butyric acid (not investigated in germ layers and some of the ova studied)
serine		

(1) Phenylalanine and the leucines could not be resolved for identification in the phenol-collidine-lutidine system.
(2) α-amino-n-butyric acid was found only in basic hydrolysates.
(3) Glutamic acid, methionine and threonine were subjected to barium hydroxide hydrolysis under conditions identical with those used in the experiments and were not found to yield a substance similar to α-amino-n-butyric acid on subsequent chromatography.

TABLE III

Differences in ninhydrin reactive substances found in hydrolysates of several species already listed

	Glucosamine(?)	Taurine(?)	Spot 1	Spot 25
Sun fish	+	+	+	—
Sea urchin	+	+	—	—
Killifish	+	+	—	—
Etheostoma	+	—	—	—
Chicken embryo	+	—	—	—
Brine shrimp	+	—	+	—
Crepidula fornicata	—	—	—	+

complement of amino acids identified in ovarian egg hydrolysates was found in the non-protein nitrogen fraction in the form of free amino acids. Essentially similar results have been found in studies of larvae (6 mm) extracts. Aspartic acid, glutamic acid, serine, glycine, alanine, valine, leucine, tyrosine, "fast arginine" and a number of unknown ninhydrin reacting components, presumably peptides and amino acid derivatives, have so far been detected. Among these and existing in relatively high concentration was a molecular species which became localized in the paper chromatogram under glutamic acid (Fig. 1). A species of similar properties has been observed by Roberts and Tishkoff (5) in their study of the normal epidermis of mice and, to a much greater extent, in epitheliomas derived from this epithelium. It seems of considerable interest to pursue the identification of this compound, keeping in mind its possible relation to the rapid growth characteristic of neoplasms in general.

III. Finally, a survey of the amino acid composition of the ova of different vertebrate and invertebrate species was made. Due to difficulties in transit, a few of the marine forms were preserved in 70 per cent ethyl alcohol instead of preserving them by the customary freezing method. Again, no appreciable differences in amino acid composition were found among all the egg species studied. The results of these and of the preceding experiments are summarized in Table II. In addition to the amino acids found common to the material studied, some species contained detectable amounts of glucosamine, taurine, and a number of as yet unidentified substances as shown in Table III. Spots 1 and 2 of Fig. 1 refer to two of these unknown substances taking up characteristic positions on the composite chromatogram.

SUMMARY

Many known amino acids have been found to occur free in very low concentration in ovarian frog eggs and larvae. In addition, several as yet unidentified ninhydrin reactive molecular species have been observed; of these, both "underglutamic acid" and "fast arginine" occur in relatively high concentration.

The data obtained on hydrolysate studies indicated that there is a surprising similarity in the total amino acid composition of all the egg types, developmental stages and germ layers investigated (Table II). In only a few species specific differences in the distribution of ninhydrin reactive substances were encountered (Table III). As far as the amphibian egg is concerned, the chromatograms give no indication that new amino acids appear and others disappear in the course of embryonic and larval development. Those of the amino acids which were approached with semi-quantitative methods were found to be present in all of the tissue primordia of the amphibian embryo without showing appreciable local differences of concentration (Table I). Therefore, if there is to be an embryonic chemodifferentiation within the cellular protein components, it apparently does not occur at the level of the amino acids, but on a higher plane of molecular organization which still eludes a biochemical approach. Evidently, the egg receives from the maternal body a vast array of amino acids ready made, and the subsequent embryogenetic elaboration of structurally and metabolically specific proteins results from specific integrations and regroupings of these units rather than from their neo-formation.

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THE BEHAVIOR OF CHROMATIN IN NORMAL AND PATHOLOGICAL HUMAN BLOOD CELLS

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INTRODUCTION

The in situ methods available for the study of cell nucleoproteins, are both physical and histochemical. Among the physical methods, the one evolved by Caspersson (9), permits of the measurement of the nucleic acids, either when free, or when combined as nucleoproteids of the protoplasm.

This method utilizes the difference of absorption maxima in ultraviolet light, of the proteins and of the nucleic acids.

The histochemical methods in general use, are based on the colour reactions obtained by the combination of some substances with the single components of the nucleotides (P of phosphoric acid, aldehydes of pentose, purinic or pyrimidic compounds). Krugeli's method (16) is based on the principle (Gomori) that phosphatases can, in tissues fixed with alcohol, hydrolyze a phosphoric ester which in turn, in the presence of Ca, becomes Ca phosphate.

The presence of the phosphate is shown by treating with cobalt nitrate or potassium sulphide. Another method is the Feulgen reaction which utilizes Schiff's reagent (unstained basic fuchsin with SO_2); the colour being recovered in the presence of an aldehyde group (desoxyribose).

Some authors (Stedman (23), Carr (7)) have expressed their doubts concerning the specificity of Feulgen's reation; but Brachet's researches (6) show that this method is specific for thymonucleic acid.

A method for detecting ribonucleic acid in the cells, is that developed by Brachet (4) which integrates Unna-Pappenheim's staining technique with treatment with ribonuclease. The latter hinders staining with pyronine which usually stains pink. Both nucleolus and cytoplasm stain richly for ribonucleic acid.

More recently, Mirsky and Ris (18) have succeeded in showing that the actual quantity of thymonucleic acid is constant in different cells of the same organism, when the chromosome number is constant. Consequently, quantitative variations of the same substance should only be due to variations in the protein associated with thymonucleic acid. These observations make us cautious about interpreting differences in staining, as differences in nucleic acid content. This investigation aims at an analysis of cell structure during the resting stage and during division into different blood diseases, such as secondary anaemias, pernicious anaemias, leukaemias and diseases connected with possible alterations of the marrow giant cells.

MATERIALS AND METHODS

In a previous series of researches on morphological aspects of the mitotic cycle in the blood cells of some blood diseases (19, 20, 22), it was possible to show typical morphological abnormalities in bone marrow cells.

In addition to the aceto-carmin and iron-haematoxilin technique, both Feulgen's and Unna-Pappenheim's methods were employed. Dubos and Thompson's ribonuclease method was not used, because Brachet's (4) exhaustive researches which were conducted at an earlier date, have shown that the basophilic properties of blood cell cytoplasm, depend solely on the ribonucleic acid contents.

The fixatives employed were: the La Cour fluid (17) for Feulgen's method, and the Carnoy mixture, for Unna-Pappenheim's. Fixation by means of heating has also been used.

The material employed was composed of smears of bone marrow of seven normal subjects, thirty secondary anaemias, four pernicious anaemias, twenty leukaemias (among which four were acute), three polyglobulias, among which one was a Vaquez, and a small but heterogeneous group of tumours.

OBSERVATIONS

A. *Erythroblastic series in normal and in secondary anaemias*

The haemocytoblast (Ferrata), stained with the usual dyes, shows the typical characteristics which permit a clear diagnosis. The cytoplasm, which can be stained a pale pink with pyronine, has a rather homogeneous appearance, while the nucleus reveals a very slightly colourable chromatin, when treated with acetocarmin, iron-haemotoxylin, Feulgen's reaction or methylgreen.

The chromonemata appear to be condensed into a very thick ball of filaments. There are single chromonemata where small chromomeres are distinguishable. The nucleolus or nucleoli are Feulgen-negative, and assume

a pink colour with pyronine. A small chromatic thickening of variable thickness which is strongly Feulgen-positive, and also strongly stainable with aceto-carmin and haematoxylin is visible around the nucleoli.

The evolution of this element towards the red series, is proved by many essential facts. The cytoplasm assumes a characteristic dark red colour which makes it stand out from the elements evolving towards the white series. The outstanding pyroninophilia of the cytoplasm (Ferrata's paradoxical phenomenon) indicates a remarkable increase of cytoplasmic ribonucleic acid. Parallel to these modifications, the nuclear chromonemata become more evident, and form areas of Feulgen-positive chromatic agglomerations which appear precociously, and can be easily distinguished (Fig. 1). The strong stainability of the cell with Pappenheim's fluid, and of the nucleus with Feulgen's reaction, now clearly differentiates the elements of the red series from those of the white one. This distinction also applies to the cells in mitotic acitivity. With the appearance of haemoglobin, the cytoplasmic ribonucleic acid begins to disappear gradually; so that finally, there is no trace of these substances in the erythrocyte.

It is evident from the previous description, that the first step linking the haemocytoblast with the red series, is the increase in staining capacity for ribonucleic cytoplasmic acid; and the appearance of Feulgen-positive nuclear masses, following the disappearance of the nucleolus. This increase in staining capacity, marks a rise in nucleic acid which, according to Caspersson (9), is the expression of an active protein metabolism. It appears also to be closely connected with the increasing mitotic activity of the red series of elements. The chromosomes are deeply and compactly coloured by Feulgen's reaction, and are short and thick (Fig. 2). In the metaphase of the haemocytoblast, the chromosomes are regularly distributed in a hollow spindle, and well disposed on the equatorial plate.

Morphological studies of the chromosomes prove rather difficult, since it becomes increasingly hard to pick out distinct chromosomes, as the metaphase proceeds towards polychromatophilism. In fact, even with suitable cytologic methods (aceto-carmin and iron-haemotoxylin) exact observations are not possible. Morphological abnormalities, observed in anaemias, do not appear to us as specific; and differ only in their frequency, when compared with other diseases. The most frequent abnormalities are the bridges in the anaphase. In agreement with Fieschi (13), and in opposition to the statements of La Cour (17) and Kienle (14), this writer can assert that the presence of multipolar spindles with the production of hypoploidal cells is uncommon.

Sometimes mitoses show overstained and remarkably contracted chromosomes, and even a granular or vescicular aspect (Fig. 3). Consequently, this morphological appearance should not be considered as the result of pycnosis phenomena during mitotic activity.

The observed abnormalities concern both metaphase and anaphase. Since the telophase is small and occurs infrequently on the observed slides, it cannot be considered sure that these abnormalities concern this phase as well. From the facts stated above, it is evident that the first histochemical modification of the haemocytoblast which evolves towards the red series, consists of an increase of stainability of cytoplasm, and possibly of nuclear thymonucleic acid; if the presence of stainable masses in the resting nucleus, is accepted as evidence. However, this is only a question of interpretation. The subsequent loss of ability to divide, remains an open question. Two possible explanations may be considered: the loss may be attributed to the mitotic abnormalities, or to the appearance of haemoglobin in this series of cells.

B. *Megaloblastic series*

The cytoplasm of the megaloblast, with the exclusion of the relative modification of the nucleocytoplasmic ratio, does not seem to differ from the normoblast. It is possible to evaluate some differences in the nucleus. Chromonemata, in the immature phases, are slightly stainable with Feulgen and other stains, and constitute a very dense mass. In the chromonemata, large chromemers are distinguishable, which are connected with one another by thin lines (compare the drawings and microphotographs in my paper on pernicious anaemias, 1946). In the perinucleolar zone, the small compact Feulgen-positive masses appear more clearly than in the normoblastic series. Also here, the disappearance of the nucleolus coincides with the increase in Feulgen-positive masses.

The characteristic features observed in this cellular type during mitotic activity, are very clear. In megaloblastic series, it seems appropriate to classify the mitoses in two groups: mitoses of the basophilic, and mitoses of the polychromatophilic series.

The megaloblastic prophases, which are notably frequent, are typical examples. In an earlier paper on these elements, the present writer observed that their prophases differ from those of the normoblastic series, with respect to the size both of the chromonemata and of the chromosomes. In fact, in the megaloblastic element, the chromosomes are longer and thinner. At the time, this could be possibly considered due to a coarser coiling of the

chromatic fiber. New evidence is provided by the Feulgen reaction. The prophase of the megaloblastic elements (promegaloblasts and basophilic megaloblasts) is stained less intensely and less clearly than the corresponding elements of the normoblastic series.

This finding is more evident in the typical and frequently occurring metaphases, where the chromosomes are long and slender.

In metaphase plates seen from the pole, the clearly distinguishable chromosomes are present in diploid number. The morphologic aspect of the chromosomes have already been described; here it can be added that these chromosomes are stained by the Feulgen reagent in a faint and uniform manner. To make a scale of comparison, these elements should be classified between the strongly Feulgen-positive elements of the erythroblastic series, and the less stained chromosomes of the white series. The scarce polyploid metaphases already found, do not differ in the morphological characteristics of the single chromosomes, from those previously described.

In a previous investigation, this writer was unable to observe the characteristics of the mitoses of polychromatophilic and orthochromic elements. At this stage, the chromosomes lose the attributes previously described, owing to two modifications of special interest. In fact, they become strongly Feulgen-positive, and assume the thick aspect of chromosomes in the erythroblastic series. Also in these megaloblasts, the chromosomes seem to have increased their coiling; and owing to modifications inherent in the spindle they come in contact with, when lying in the metaphase plate, it becomes difficult to analyze them with accuracy.

The increased rapidity of cellular division is noticeable by the greater number of elements in mitotic acitivity, and by the presence of tripolar telophases. Multipolarity causes the formation of hypoploid cells; and in some metaphases where counting is possible, hypoploid cells are found. Microphotographs showing these phenomena, appeared both in La Cour's and in previous papers by this author. These alterations of the orthochromic series have been described by Fieschi (13).

According to Fieschi, modifications in number are only apparent; other phenomena are to be interpreted as pycnosis which begins in elements during mitosis, after the appearance of haemoglobin. A more suitable technique enables us to consider the variation in number as a real phenomenon.

C. *White series* (*normal marrow, anaemias and leukaemias*)

The elements evolving towards the white series, differ from those evolving towards the erythroblastic series, in many aspects. In the Unna-Pappen-

heim method, the cytoplasm stains a pale pink, and contrasts sharply with the dark red colour of the cytoplasm of the red elements. In the nucleus, it is observed that the chromonemata of the cells, during evolution towards the white series, become less tangled, though still retaining the aspect of intermingled threads; so that the nucleus appears to have many empty spaces. The chromonemata are always distinguishable from those of the red series, because they are less strongly coloured, and the chromatin clumps are less clear, as will be seen later, at a further stage of their evolution. In the myeloblastic series, in the proximity of the nucleoli, the compact perinucleolar chromatic thickenings are also visible, and become strongly coloured when treated with Feulgen's reagent and the other two nuclear dyes.

The nucleoli assume a red colour when stained with Unna-Pappenheim's reagent and this differentiates them from the cytoplasm. The difference is due to the greater uniformity of the latter. The cytoplasm of the promyelocytes is characterized by a pink colour; while on the outer area, bright red granules are apparent. These granules are particularly clear in the eosinophilic myelocytes.

In the late stage of white cell development, staining intensity decreases progressively, and only a pale pink persists.

Although the action of ribonuclease has not been tested as described by Brachet, his evidence enables this author to interpret the strong stainability with pyronine, as an indication of ribonucleic acid in the cytoplasm. This staining also remains in the ripe granulocyte; this does not hold good in the case of the erythrocytes.

I was unable to detect a single constant abnormality which would make it possible to differentiate the leukaemia cells during the resting stage, from normal ones.

During mitotic activity, the cells deserve careful consideration. The mitoses of the myeloblasts and myelocytes treated with aceto-carmin and haemotoxylin, do not present typical divergences from the normal line. The chromosomes of the white elements, stain much less intensively than those of the red elements. They become slender, and are, as a rule, regularly disposed in the equatorial plate; and it is therefore possible to determine their number. These are diploid. Anaphases and telophases occur infrequently. Sometimes long chromosomes show a tendency to remain in terminal contact during anaphase. The possibility of a false bridge is open; but no decisive evidence can be presented (Fig. 4).

In a severe secondary anaemia, following gastric carcinoma, an abnormal behavior of the myelocyte mitotic cycle was observed. In fact, typical

prophases and prometaphases were present in considerable numbers (Figs. 5, 6, 7). It was not possible to observe any well defined stage of equatorial plate and anaphase.

Endomitosis is generally characterized by an incomplete cycle. However, no polyploidal numbers were ever observed and can, consequently, lend no definite support for endomitosis.

The single chromosomes were not different from the normal ones. The Feulgen reaction shown by the chromosomes, both of the white series of normal marrow, and of the anaemias under consideration, reveals a feature of some interest. These chromosomes stain so faintly, that they could be defined as having a low thymonucleic acid content. In fact, there is a sharp contrast here, as compared with the red cells during maturation (Fig. 8). The collected data are similar to the quantitative findings of Thorell (21). According to him, there should be a notable difference between the thymonucleic acid of the white and red series. A relationship appears to exist between reactivity with Feulgen staining, and the thickness of the chromosomes; it is strong in the latter, where the staining is stronger; and weak, where the reaction is also weak. Therefore it was possible to confirm La Cour's (15) conclusion, that there is a close relationship between: "little ribonucleic acid in the cytoplasm, little thymonucleic acid in the chromosomes, and the presence of a hollow spindle."

As for the mitoses in leukaemia, the abnormalities described in four cases of acute leukaemia (two cases have already been described as part of a group of twenty leukaemias) may be briefly summarized as follows: 1) prophases with the persistence of the nucleolus with a reduced chromosome number; 2) metaphases which presented, in three cases, a number of chromosomes varying below the diploid number (while in one case, haploid

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Fig. 1. Nucleus of erythroblast with zones of chromatin clumping. Microphot. 1,500 diam. approx. Staining iron-haematox.

Fig. 2. Chromosomes of erythroblast strongly stained with Feulgen-reactive. Microphot. 1,500 diam. approx. Staining Feulgen.

Fig. 3. Chromosomes of erythroblast strongly stained and spiralized. Microphot. 1,500 diam. approx. Staining aceto-carmin.

Fig. 4. Anaphase of immature element of the white series. Chromatin bridge. Microphot. 1,500 diam. approx. Staining aceto-carmin.

Fig. 5, 6, 7. Prophase and prometaphases of unripe elements of the white series. Microphot. 1,500 diam. approx. Staining iron-haematox.

Fig. 8. Element of normal myelocytic series, scarcely stained with Feulgen reaction. Microphot. 1,500 diam. approx. Staining Feulgen.

Fig. 9. Equatorial plate of white leukaemic element, recalling a meiotic stage. Microphot. 1,500 diam. approx. Staining iron-haematox.

Fig. 10. Metaphase of leukaemic element strongly Feulgen-positive. Microphot. 1,500 diam. approx. Staining Feulgen.

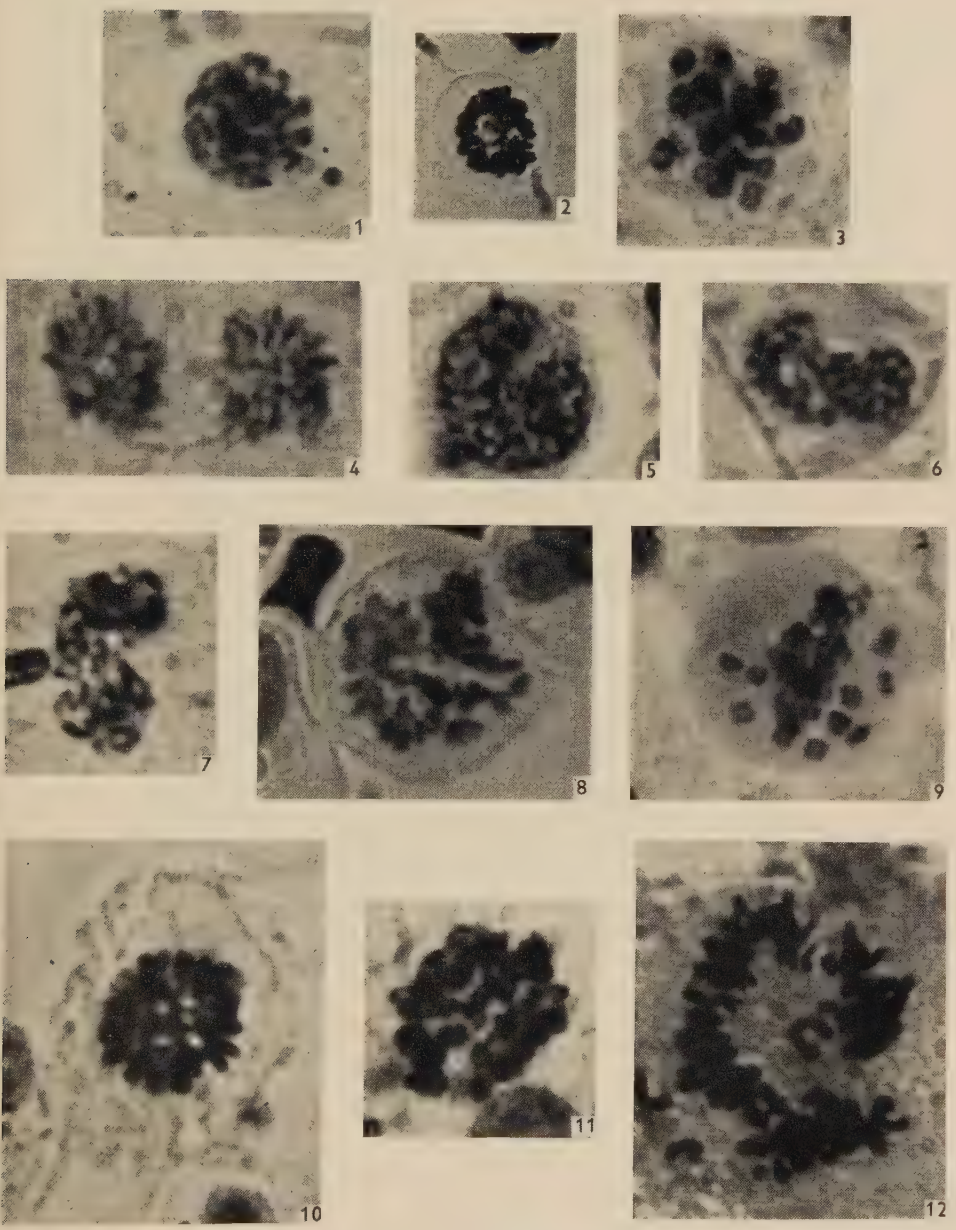
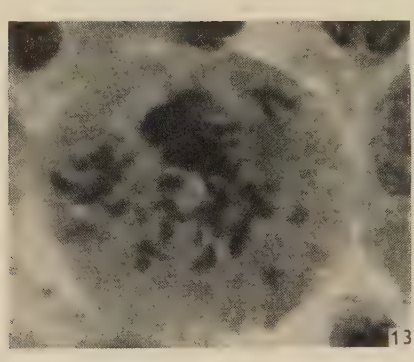
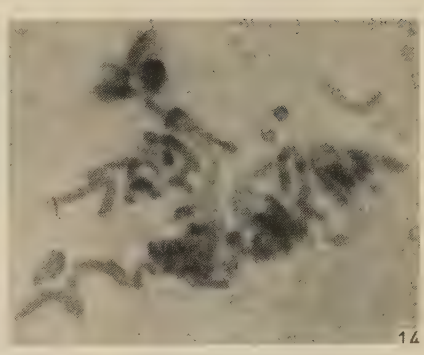
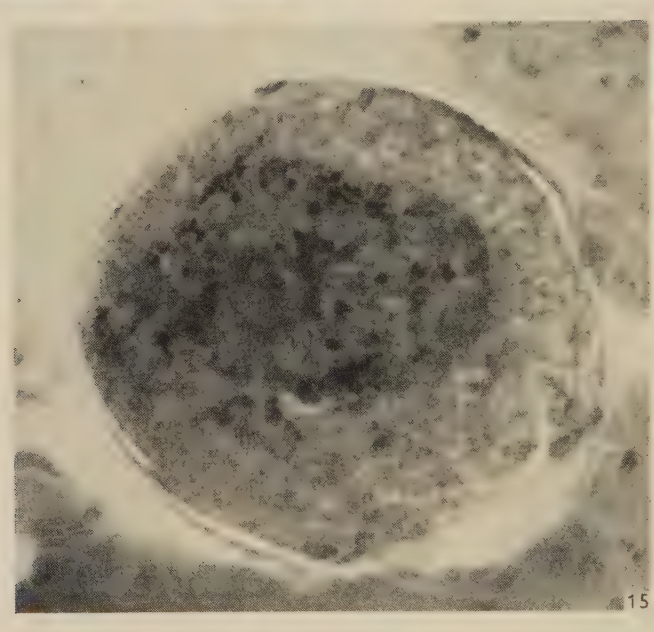


Fig. 11. Metaphase of normal lymphoblast. Microphot. 1,500 diam. approx. Staining iron-haematox.
Fig. 12. Hyperploid metaphase. Microphot. 1,500 diam. approx. Staining iron-haematox.







Figs. 13, 14. Asymmetric figures. Microphot. 1,500 diam. approx. Staining aceto-carmin and iron-haematox.

Fig. 15. Giant marrow cell. In the nucleus several small nuclear masses strongly stained with pyronine, are visible. Microphot. 1,500 diam. approx. Staining Unna-Pappenheim.

sets were also observed); 3) telophases with polar groups in different stages of despiralisation; 4) the presence of some equatorial plates, with thick chromosomes in pairs, recalling a mature meiotic stage (Fig. 9).

When using Feulgen and Unna-Pappenheim's staining methods, it was observed that elements in mitotic activity, the chromosomes of leukaemic

elements, stain strongly with the Feulgen reaction (Fig. 10). This is distinctly not the case in the normal series (Fig. 8). This observation applies most particularly to the myeloid leukaemias. The element of the lymphatic series presents, already at the normal stage, a rather thick and strained aspect; and consequently this difference is not so great (Fig. 11).

The variations of the mitotic picture described above, and the abnormalities of the typical staining for nucleic acid, seem to become manifest at the commencement of leukaemia.

RELATIONSHIP BETWEEN LEUKAEMIA AND TUMOURS

For a discussion on the relationship existing between tumours and human leukaemia, it will be found useful to compare the cell structure, already described, with a tumour of the pleura. This tumour may be used to provide a good example of neoplastic deviation from endothelial cells. In the tumour in question, one sees, in addition to many normal pictures, hyperploid metaphases (Fig. 12), and metaphases with displaced chromosomes having spindle errors (Figs. 13, 14). It is evident that these phenomena correspond mainly to those found in leukaemia. It is well known that many investigators have tried to compare leukaemia with neoplasias; and they have succeeded in finding some similarities. If the problem is also considered as a cytological phenomenon, then the observations carried out on the tumour cells may be summarized by differentiating the structure of the proliferating cells during the resting stage (A cells according to Caspersson and Santesson (11)), from that of degenerating cells also at the resting stage (B cells according to the same authors). Furthermore, some mitoses are abnormal for different characters, and some metaphase-chromosomes are overpacked (Koller (15), Cusmano (12)). Of these considerations, only the latter, i. e. the behavior of chromosomes during mitosis, is comparable to my findings for leukaemia. No similarity has been found to exist with regard to the nuclear structure during the resting stage.

D. *Megakaryocytic series*

It has been stated (Polli 22) that in several blood diseases, the megakaryocytic series also displays a complete normal mitotic cycle. The chromosome number is very high and exceeds the normal diploid. Some spindles are multipolar. One aspect of the giant cells during the resting stage could be demonstrated by means of the Feulgen reaction and Unna-Pappenheim's

staining. In the polymorphic nuclei of such cells, among chromatin colored in methylgreen, the presence of numerous small compact masses, colored bright red with pyronine, and strongly Feulgen-positive, was evident. The small masses cannot be considered as being nucleoli, because of their number and shape (Fig. 15). As distinct from other myeloid cells, these masses are rather numerous, even during the youngest stage of the cells, when the nucleoli were still present; while later, they are known to disappear. The abnormalities of these cellular series, so typical among the others, can be summarized as follows: the presence of a high degree of polyploids and of many chromocenters which are strongly Feulgen-positive. These chromocenters are also deeply stained with Unna-Pappenheim's pyronine; so that they indicate a notable quantity of ribonucleic acid content. The same phenomenon was not observed in the highly unripe elements of other cellular series.

DISCUSSION

The investigations carried out by Caspersson and his co-workers (10) have clarified the phenomena of nucleic acids. At a later date, Mirsky and Ris (18) demonstrated, when employing other methods, that thymonucleic acid varies only in the percentage of the total mass (nucleic acid + protein) contained in the nucleus. We have now to proceed with the morphological and structural work, in order to describe the finest variations, both of chromatin and cytoplasm, in connection with the phenomena referred to above.

The latter object has been investigated in the classical researches. The study of chromatin structure was started in connection with normal cells (see Barigozzi (1)); later, malignant cells were also studied in this connection (Koller (15), Cusmano (12), Barigozzi and Casabona (3), Casabona (18)). At present, it embraces both normal and pathological blood cells.

The usefulness of such investigations is suggested by the possibility of finding in the chromatin of different kinds of cells, structural differences of a particularly delicate character, but which are fixed for every species and condition of cell. It is known that the distribution of chromatin in a nucleus, is not the expression of the primary condition of the genome (which mainly functions during differentiation and development); moreover, it is known that the characteristics displayed by the chromatin (its content of nucleic acid, the presence of absence of the nucleolus) are important tests in order to show whether a cell is capable of undergoing mitosis, or if endomitoses occur. All these applications of cytogenetical concepts to the soma-

tic cells (whether normal or pathological) should be fruitful for the blood cells, as they have been for the malignant elements.

Although the observations recorded in the present paper may not be of diagnostic value, they may, however, serve as a starting point for clarifying the problem of the relationship which exists between neoplasias and leukaemias. As seen in the nuclear structure during the resting stage, this relationship does not appear to be a close one.

GENERAL CONCLUSIONS

1) Red cell series

   a) Normoblastic series:

   During the evolution of the primordial element towards red cell, the following observations have been made:

   i) The cytoplasmic stainability with pyronine is very strong.
   ii) The precocious appearance of Feulgen-positive masses within the nucleus.
   iii) Mitoses with chromosomes strongly Feulgen-positive.

   b) Megaloblastic series (pernicious anaemias):

   i) The behavior of cytoplasm is identical with the former case.
   ii) The mitoses are pale when stained with Feulgen reaction, and are characterized by slender chromosomes (initial phase of maturation), or by contracted and strongly Feulgen-positive chromosomes (polychromatophilic phase).

2) White cell series

   a) Normal white series:

   During evolution from primordial elements towards the white cell, the following observations have been made:

   i) Moderate stainability of the cytoplasm with pyronine.
   ii) Weak stainability of the chromosomes with Feulgen reaction.
   iii) Mitoses with hollow spindle.

   b) Leukaemic series:

   i) The cytoplasm behaves as in the white normal series.
   ii The chromosomes are, contracted, strongly Feulgen-positive, and seem often to be paired.
   iii) The mitotic cycle is often abnormal, with varying chromosome number.

3) Megakaryocytic series (Giant marrow cells)

i) In the resting stage, the nucleus displays many chromocenters, both Feulgen and pyronine positive.

ii) The mitoses show hyperploidy, and the spindles are sometimes multipolar.

I wish to express my thanks to Prof. Fasiani (Clinica Chirurgica della Università) and to Prof. Bucalossi (Istituto del Cancro di Milano) for their kind help in providing tumour material.

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OBSERVATIONS UPON THE RESTING NUCLEUS OF THE TYPHOID BACTERIUM

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INTRODUCTION

The form of the nucleus of young, vegetative cells of bacteria is now well known. The information upon the subject has been fully discussed by Piekarski (4) in an admirable review, and in a monograph (2) by the author of this paper. It has been suggested by the author (1, 2), that this vegetative nucleus, with its rod-shaped chromosomes, or chromosome complexes, is in a semipermanent mitotic condition. A vesicular nucleus is found in the microcyst stage, which is adopted by resting cultures of almost all bacteria, as well as in the better known spore of *Bacillaceae* and microcyst of Myxobacteria.

This microcyst is spherical or oval in shape, and its morphology is very typical, so that the genera of the otherwise morphologically indistinguishable *Bacteriaceae* may thus be distinguished (3).

The microcyst of *Salmonella typhi* is especially large, and the structure of the nucleus can readily be discerned by both electron and photomicroscopy.

METHODS

A strain of *S. typhi* was used in which the maturation of the microcyst was very rapid, being complete after two or three days. Preparations were stained by the HCl-Giemsa technique. Wet smears were fixed in osmic acid vapour, stained and mounted in water. The degree of acid-hydrolysis required to reveal the nuclear structures of the resting cells was considerably less than was required by the corresponding vegetative cells. This was due, presumably, to their lower nucleotide content. (For techniques see (2)).

Preparations for electron microscopy were thoroughly washed in saline and then in distilled water. This method was successful in reducing the opacity of the cells to the electron beam by the removal of surface mucus. Metallic shadowing was not employed.

OBSERVATIONS

With the microscope the resting cell of *S. typhi* was clearly seen to possess a large, central nucleus, with a well-marked vesicular structure, a nuclear membrane and an eccentric chromatin granule occupying about one third of the total volume of the nucleus (Figs. 1 & 2).

This vesicular appearance was also seen quite clearly by the electron microscope (Fig. 3), but the details of the structure of the nucleus were not so well shown, despite the better definition of the electron microscope. The unavoidable desiccation of the material, in preparation for the vacuum chamber, was presumably responsible for this defect. The lower cell in Fig. 3 is in the vegetative condition. The two, transverse nuclear elements can be distinguished, although their outline is not as distinct as in the resting cells.

SUMMARY

By the electron and light-microscopes the resting nucleus of *S. typhi* is shown to possess a well-marked vesicular structure, with a nuclear membrane and an eccentric granule.

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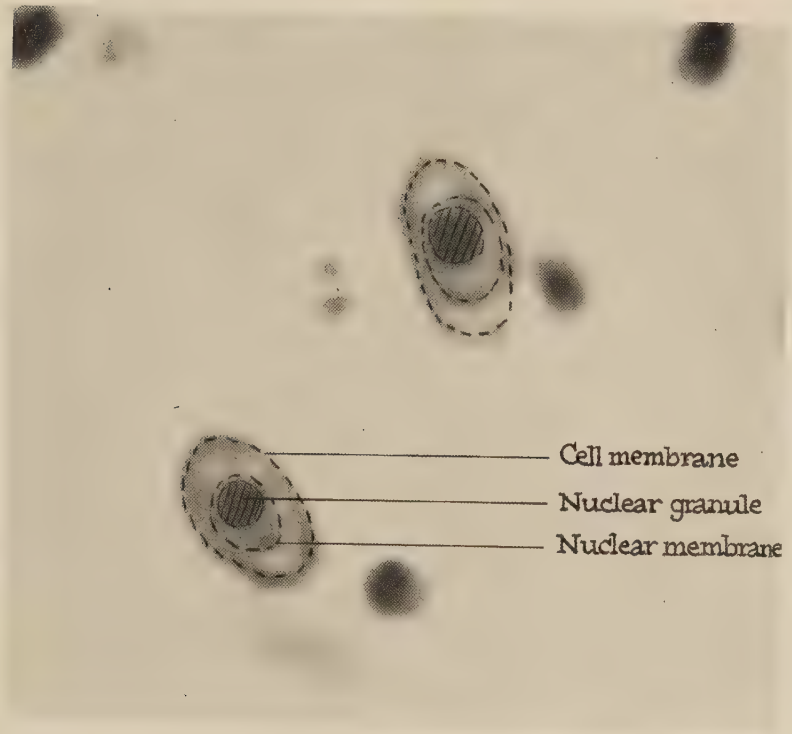

Fig. 1.

Fig. 2.

Fig. 1. *S. typhi.* Microcysts, stained by acid-Giemsa, the main structures outlined. ×7,500.
Fig. 2. The same as 1, without outlining. ×5,000.

(The electron micrograph was made with the kind assistance of Miss C. M. F. Hale and Mr. C. C. Newton).

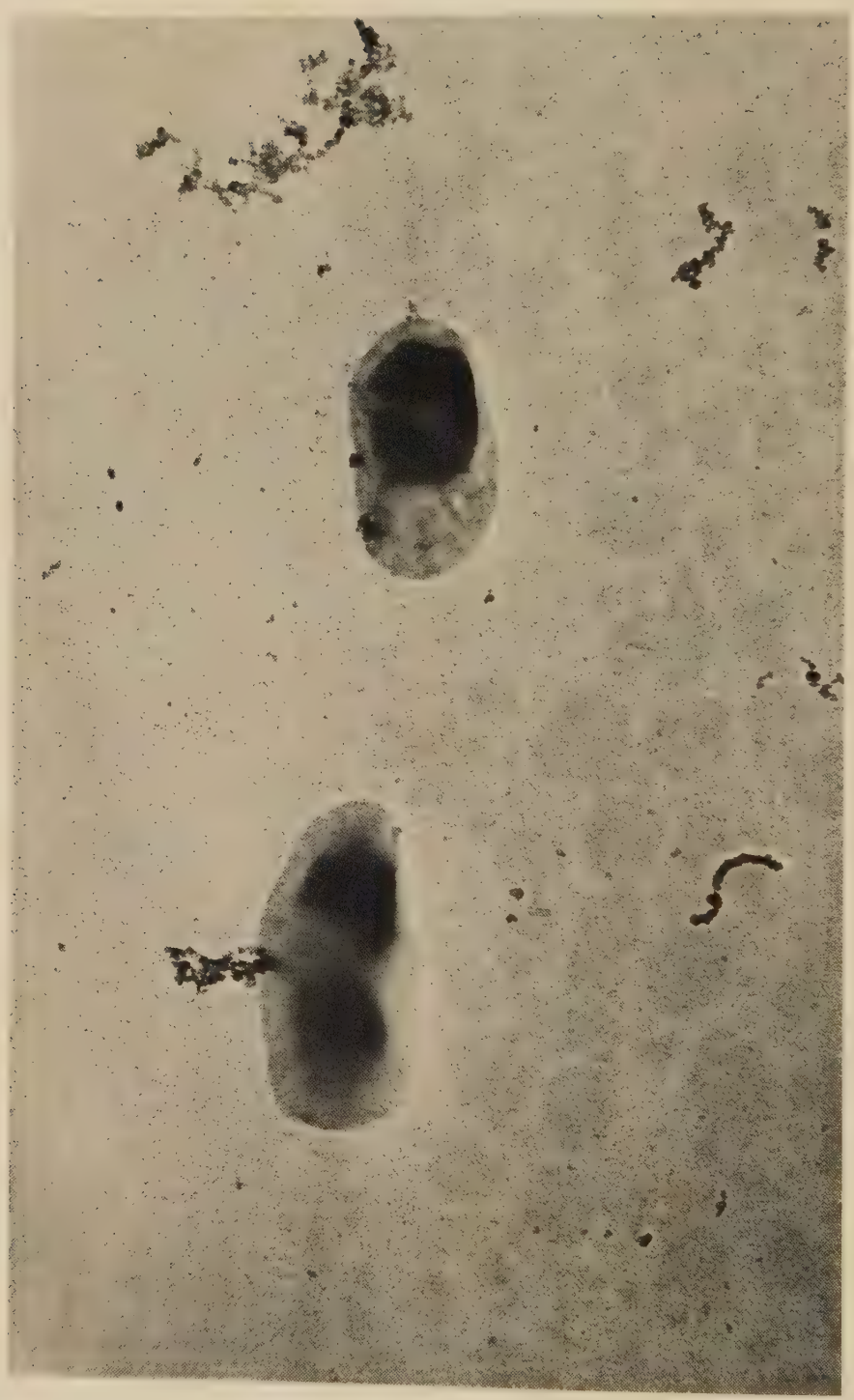

Fig. 3.

Fig. 3. Electron micrograph. Above, microcyst showing vesicular nucleus; below, vegetative cell showing chromosomal nucleus. ×16,000.

DIFFERENTIAL STAINING OF VARIOUS POLYSACCHARIDES IN SEA URCHIN EGGS

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Metachromatic staining of the cytoplasm has been observed in the cells of several tissues which were fixed and stained with suitable basic dyes (6, 7, 8, 25, 26, 28, 29). Holmgren, Holmgren and Rexed and Sylvén arrived at the conclusion that this staining is due to sulfate-containing mucopolysaccharides present within the cytoplasm. Moreover, it has been observed by Harvey (5) that the ground cytoplasm of living sea urchin eggs stratified by means of centrifuging is stained "pinkish-lavender" with toluidine blue, which fact means that in this case metachromatic color shift was also obtained. Runnström (22) advanced the view that a heparin-like sulfate-containing mucopolysaccharide present within the cell is able, under certain conditions, to inhibit the enzymes which are responsible for protoplasmic clotting. In an earlier paper Runnström, Tiselius and Lindvall (20) claimed that the physiological "liquefaction" of the protoplasm may be due to a detergent-like substance and Monné (15) and Runnström (21), supposed that this detergent-like substance may be responsible for the dissolution of various protoplasmic structures during mitosis and the normal functioning of the cell. Later on Monné (16) advanced the hypothesis that mucolipids are obligatory constituents of the protoplasm, and that these mucolipids are able, under certain conditions, to act like detergents because they appear to be polar-nonpolar compounds. One may remember that bacterial mucolipids are powerful physiological agents able to stimulate, injure and destroy the cells of the host and that these mucolipids lose their toxicity when split into their component parts; lipids and polysaccharides (14). Gersh (3) demonstrated that the Golgi-apparatus contains a protein and a carbohydrate (not glycogen) and Monné (16) supposed that this Golgi-apparatus consists of a mucolipid in association with a protein.

The condition of the mucolipid seems to be responsible for structure and

physiological properties of the protoplasm. In stimulated cells the structure of the cytoplasm is fine (for details and literature see Monné, (17)) because the mucolipid is bound to the cytoplasmic fibrils by means of its carbohydrate part or because the hydration of the carbohydrate part of the mucolipid bound to the cytoplasmic fibrils is increased. In excessively stimulated cells the structure of the cytoplasm is still finer because the mucolipid of the Golgi-apparatus is dispersed (16 p. 120) and bound to the cytoplasmic fibrils. Upon inhibition the structure of the cytoplasm becomes coarse because the mucolipid is separated from the cytoplasmic fibrils or because the hydration of the carbohydrate part of this mucolipid is decreased.

Thus, it is more and more apparent that polysaccharides and other carbohydrates are of great importance for the functional condition and for the structural transformations of the cell. For this reason it was interesting to investigate the properties and the distribution of the protoplasmic polysaccharides. The chemistry of the jelly coat of the sea urchin eggs has been carefully studied by Vasseur (27) and Immers and Vasseur (10). Monné (16) has found by means of microchemical methods that at least two different polysaccharides are present within these eggs. A method by which different polysaccharides are stained in different colors is reported in this paper.

Carbohydrates can be stained with all the methods which are used for detecting aldehydes when the aldehydes are liberated from the carbohydrates under the influence of suitable oxidants, particularly periodic acid. The reaction brought about by periodic acid is most valuable because it is highly specific. Upon treatment with periodic acid, carbohydrates are stained violet by means of fuchsin-sulfite (Hotchkiss method) and yellow by means of 2-,4-dinitrophenylhydrazin (Albert and Leblond method). For details concerning these methods the reader is referred to the book of Glick (4). Some polysaccharides are stained yellow and others are stained violet when these two methods are combined with one another. The principle of this combined method is explained below.

Oocytes, mature unfertilized, fertilized and cleaving eggs, blastulae, gastrulae and plutei of the sea urchin *Paracentrotus lividus* fixed in Bouin fluid were chiefly used for the staining experiments. Eggs and embryos of other sea urchin species were also used, but to a minor extent. The eggs are well fixed when immersed in Bouin fluid in isolated condition. They are badly fixed when parts of ovaries are immersed in this fluid. This occurs even when the parts are small (16). The eggs were fixed in the ordinary Bouin fluid because it was not important for the present investigations to preserve glycogen, on the contrary it was desirable to dissolve this substance,

because the purpose of these investigations was to show how polysaccharides, other than glycogen, particularly mucopolysaccharides, are distributed within the egg. Nevertheless, Bouin fluid is not an ideal fixative because it is able to preserve the mucopolysaccharides to a large extent, but not completely. Particularly the acid mucopolysaccharides are incompletely fixed or dissolved. Thus, it was found that the jelly coat of the eggs is dissolved. This jelly coat could be preserved only when precipitated in some way prior to fixation (16). The more hydrated a polysaccharide is, the more difficult it is to preserve. There is no doubt that the hydration of the protoplasmic polysaccharides is subjected to considerable changes during the functioning of the cell.

The combined staining procedure with fuchsin sulfite and 2-,4-dinitrophenylhydrazin is as follows:

1. Run paraffine sections through xylol and alcohols to distilled water.
2. Place in the periodic acid solution described by Hotchkiss (9) for 10–15 minutes at room temperature.
3. Rinse thoroughly in distilled water which is changed several times.
4. Immerse in 70 per cent alcohol.
5. Place in the 2-,4-dinitrophenylhydrazin reagent of Albert and Leblond for 12–36 hours at room temperature.
6. Rinse thoroughly in 17 per cent alcohol which is changed several times.
7. Immerse in distilled water.
8. Place in the Schiff reagent (fuchsin-sulfite) as recommended by Hotchkiss or Feulgen for 30–45 minutes.
9. Wash 3 times with SO_2-water as described by Hotchkiss or Feulgen.
10. Immerse in distilled water, run through alcohols to xylol and mount in balsam as usual.

Excellent staining results were obtained. All carbohydrate-containing cellular components are stained violet when treated with fuchsin-sulfite alone (Figs. 1, 2), all these components are stained yellow when treated with phenylhydrazin alone. Some of these components are stained yellow and others are stained violet when the combined method is employed (Figs. 3, 4, 6–10). The differential staining is most pronounced when the preparations are kept in the phenylhydrazin solution for a suitable length of time. In this case the yolk is stained yellow and other cellular components are stained violet. The yolk is stained orange or red when the preparations are treated with phenylhydrazin less than 12 hours. This color appears because the yolk reacts to some extent with both reagents; fuchsin-sulfite and phenylhydrazin. Stain-

ing with fuchsin-sulfite, and consequently differential staining, become impossible when the preparations are kept in the phenylhydrazin solution for 2–4 days. In this case all carbohydrate-containing cellular components are stained yellow. Moreover, the cellular components which are only weakly stained when treated with fuchsin-sulfite alone, become intensely stainable by means of this reagent when pretreated with phenylhydrazin for a suitable length of time. This fact means that phenylhydrazin favors the reaction between fuchsin-sulfite and certain oxidized carbohydrates.

Preparations not pretreated with periodic acid remain unstained when subjected to the action of fuchsin-sulfite, phenylhydrazin or both. This fact proves that aldehydes are absent from the untreated sea urchin eggs, and that aldehydes reacting with fuchsin-sulfite and phenylhydrazin appear in these eggs upon treatment with periodic acid. This conclusion is also supported by the facts that staining does not appear either under the influence of fuchsin-sulfite or phenylhydrazin when the liberated aldehydes are blocked by means of sodium bisulfite and that staining with fuchsin-sulfite becomes impossible when all liberated aldehydes are bound by means of phenylhydrazin. Thus, it was found that preparations pretreated with periodic acid actually remain unstained with fuchsin-sulfite or phenylhydrazin when immersed for several hours in a 10 per cent solution of sodium bisulfite, and that these preparations, as it was mentioned above, do not stain with fuchsin-sulfite when subjected to the action of phenylhydrazin for several days.

The aldehydes which are liberated from carbohydrates upon oxidation with periodic acid can be detected not only by means of fuchsin-sulfite or phenylhydrazin, but also by means of an alkalized (ammoniacal) silver nitrate solution. The latter method was employed in the modification of Gomori (for details see 4) and excellent results were obtained. The cellular components which contain carbohydrates, particularly the yolk, are impregnated with silver and gold, and consequently appear black in the preparations.

Lipids, monosaccharides, oligosaccharides and glycogen are dissolved when the cells are fixed in Bouin fluid. For this reason the cellular components which are stained by means of the above mentioned reagents must be polysaccharides other than glycogen. This conclusion is also supported by the fact that exactly the same staining results are obtained when preparations pretreated and not pretreated with saliva are subjected to the action of fuchsin-sulfite or phenylhydrazin. It is well known that the enzyme ptyalin present in the saliva is able to break down glycogen, but not mucopolysaccharides. Thus, the carbohydrates detected within the sea urchin eggs appear

to be mucopolysaccharides or closely related hydrophilic, jelly-forming substances. It was found by Monné and Hårde (unpublished) that some of these polysaccharides are more rapidly oxidized than others by means of periodic acid. It is amazing how rapidly the polysaccharide of the yolk is oxidized by this acid. The yolk is distinctly stained by means of fuchsin-sulfite when pretreated with periodic acid for such a short time as 10 seconds, but it remains unstained when pretreatment with periodic acid is omitted. This readily oxidizable carbohydrate seems to be an aminopolysaccharide (Monné and Slautterback, unpublished). The acid polysaccharide of the jelly coat, of the blastocoelic jelly and of the hyaline layer are stained only if they are treated with periodic acid for several minutes.

All polysaccharides oxidized by means of periodic acid are able to react with phenylhydrazin, but some of them much more slowly than others. This is the reason why differential staining is produced when in the described procedure the preparation is moved at the right moment from the phenylhydrazin to the fuchsin-sulfite reagent. The polysaccharides which did not react with phenylhydrazin are able to react with the subsequently applied fuchsin-sulfite. The polysaccharides which are already combined with phenylhydrazin are, of course, not able to react with fuchsin-sulfite. All oxidized polysaccharides react with phenylhydrazin upon prolonged treatment with this reagent and, therefore, subsequent staining with fuchsin-sulfite and consequently also differential staining of various polysaccharides in the sea urchin egg become impossible.

The following staining results were obtained upon treatment of the eggs and embryos with the combined phenylhydrazin-fuchsin-sulfite method:

In the mature unfertilized eggs the yolk is stained yellow. The jelly coat when preserved (see 16, p. 106) is stained violet. The cortical granules and the coarse nodes of condensed ground cytoplasm are also stained violet, but rather faintly. Moreover, only one part of the nucleus is stained violet just as in the case of the cleaving eggs.

In cleaving eggs the yolk is stained yellow. The fertilization membrane is stained violet, and the hyaline layer (covering the surface of the cleaving egg) intensely violet. The staining of the resting nucleus is particularly interesting. Only one half of the resting nucleus is stained intensely violet, and always that half which is directed towards the centre of the embryo (Figs. 6, 7, 8, 9). The other half is unstained or only faintly colored. The division spindle, the cytoplasmic area around the poles of this spindle, and the central part of the astropheres are also stained violet (Figs. 3, 4). The chromosomes appear to be unstained.

In the blastula stage the yolk is stained yellow as usual. The hyaline layer and the jelly filling the blastocoele (see Monné and Hårde, 18) are stained intensely violet. In the early blastula the resting nuclei are stained violet exactly in the same way as in still earlier stages, that is to say only the halves of the nuclei which are directed towards the blastocoele are stained (Figs. 9, 10). The staining of the nuclei becomes gradually weaker as development proceeds. Up to this time it was impossible to stain the nuclei of fully developed blastulae, gastrulae and plutei. Moreover, in the cells of the early blastula small violet granules were found within that region of the cytoplasm which is directed towards the blastocoele (Fig. 10, 9). These granules are secreted into the blastocoele and transformed into the blastocoelic jelly.

The yolk is stained yellow in all stages: oocytes, mature unfertilized, fertilized and cleaving eggs, blastulae, gastrulae and plutei; while all other above mentioned components of the sea urchin egg are stained violet. Thus, upon oxidation with periodic acid the polysaccharides of the yolk react much more rapidly with phenylhydrazin than the polysaccharides which are associated with other components of the egg. The yolk is basic and consequently it cannot be stained with basic, but only with some acid dyes. The yolk does not contain protamines and histones because it stains with different acid dyes than the chromatin (16) which is known to contain these basic proteins in association with ribonucleic acid. The acidophilia of the yolk disappears upon deamination. The affinity of the chromidia to acid dyes also disappears upon deamination (Monné and Slautterback, unpublished), but it remains unchanged when nucleic acid is removed (Monné and Hårde, unpublished). There is no doubt that the basic properties of the chromidia which do not exhibit any strong affinity to the reagents for carbohydrates are due to the amino groups of basic proteins, and that the basicity of the yolk which exhibits a very strong affinity to the reagents for carbohydrates is not due to the amino groups of these basic proteins, but to the amino groups of some other substance, most probably the amino groups of amino sugars. Thus, aminopolysaccharides seem to be the predominant chemical constituents of the yolk substance.

The jelly coat of sea urchin eggs consists of mucopolysaccharides with sulfuric acid residues (27, 10). The hyaline layer, the cortical granules (16) and the blastocoelic jelly (18) exhibit similar staining properties to those of the jelly coat, no doubt because they also contain mucopolysaccharides with sulfuric acid residues. All these components of the sea urchin egg are never stained yellow, but always violet when the combined phenylhydrazin-fuchsin-sulfite method is applied. Thus, it is very interesting that the basic

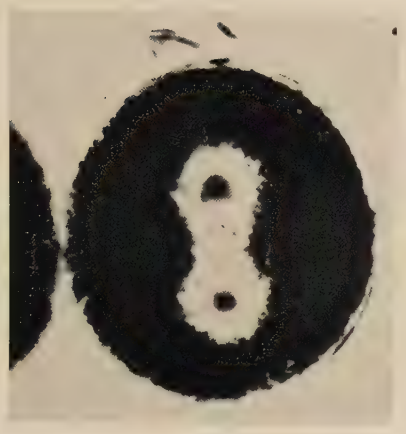

Fig. 1.

Fig. 2.

Fig. 3.

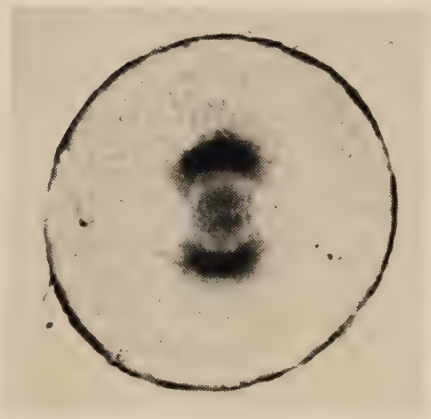

Fig. 4.

First cleavage division of the sea urchin egg.

Figs. 1, 2. Hotchkiss method. The cellular components stained violet with fuchsin-sulfite appear black in the photographs. The yolk appears in the photographs as a black, homogenous mass because the yolk globules are intensely stained and densely packed. In the corresponding preparations the yolk globules are sharply delimited from each other. The spindle is almost unstained, but the cytoplasm surrounding the poles of the spindle is distinctly stained.

Figs. 3, 4. Phenylhydrazin-fuchsin-sulfite method. The yolk which is stained yellow with phenylhydrazin appears colorless in the photographs. The cellular components which are stained violet with fuchsin-sulfite appear black or gray in the photographs. The hyaline layer, the spindle, and particularly, the cytoplasm surrounding the poles of the spindle are stained violet. The amount of the stained cytoplasm surrounding the poles of the spindle is gradually increased as mitosis proceeds. The mucoid of the ground cytoplasm appears in the form of numerous, faintly stained granules. In Fig. 3 this mucoid is condensed on the periphery where the structure of the cytoplasm is coarser than in the rest of the cell.

phenylhydrazin reacts much more rapidly with the basic yolk than with the above mentioned acid constituents of the egg.

Upon oxidation with periodic acid the blastocoelic jelly is stained quite strongly violet with fuchsin sulfite (18). The jelly is heavily stained with the same reagent (Fig. 10) when the combined phenylhydrazin-fuchsin-sulfite method is applied. The cytoplasmic areas surrounding the poles of the spindle are also stained with fuchsin-sulfite (Figs. 1, 2, Monné and Hårde, unpublished), but more intensely when the combined method is applied (Figs. 3, 4). The hyaline layer is irregularly stained with fuchsin sulfite, sometimes weakly (Figs. 1, 2), sometimes strongly (16). This layer is always intensely stained with the same reagent (Figs. 3, 4, 6, 7, 9, 10) when the combined method is employed. The spindle is faintly stained with fuchsin sulfite (Monné and Hårde, unpublished). It is colored intensely violet (Figs. 3, 4) when the combined method is applied. The nuclei of the cleaving eggs are very faintly stainable with fuchsin sulfite alone and therefore this fact was not noticed earlier by Monné and Hårde. The nuclei are stained intensely violet when the combined phenylhydrazin-fuchsin-sulfite method is applied. All these facts prove that phenylhydrazin is able to favor the reaction between fuchsin sulfite and some oxidized polysaccharides. This acceleration is important if the reaction does not proceed readily enough. Of course this acceleration is possible only if phenylhydrazin is present, but not combined with the aldehyde groups of the polysaccharide.

The resting nuclei of growing oocytes, mature unfertilized (2), fertilized and cleaving eggs are always Feulgen negative, even when treatment with hydrochloric acid is prolonged. The nuclei of the blastulae, some time before gastrulation, stain faintly, and the nuclei of the more advanced stages stain gradually stronger when the Feulgen method is applied (2). The nuclei of the cleaving eggs are able to react with fuchsin-sulfite upon treatment with

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Fig. 5. Cleaving sea urchin egg in the stage of two blastomeres stained with azocarmin G. The chromidia in the cytoplasm and the chromatin in the nucleus are stained. The chromatin is scattered throughout the whole nucleus.

Figs. 6–10. Cleaving eggs in the stage of two blastomeres (Figs. 6, 7) and blastulae (Figs. 8, 9, 10) stained by means of the phenylhydrazin-fuchsin-sulfite method. The yolk which is stained yellow with phenylhydrazin appears colorless in the photographs. The cellular components stained violet with fuchsin-sulfite appear black or gray in the photographs. Thus, the nuclear mucoid appears black. It is condensed in the halves of the nuclei which are directed towards the cleavage furrow (Figs. 6, 7) or towards the blastocoele (Figs. 8–10). The hyaline layer is stained in Figs. 6, 7, 9, 10. In Fig. 7 the nucleus is covered with an intensely stained cytoplasmic cap from which the rays of the astrosphere radiate. Granules present in the inner parts of the cytoplasm of the blastomeres are stained in Figs. 10 and 9. These granules are secreted into the blastocoele and transformed into the blastocoelic jelly. The shrunken blastocoelic jelly is intensely stained in Fig. 10.

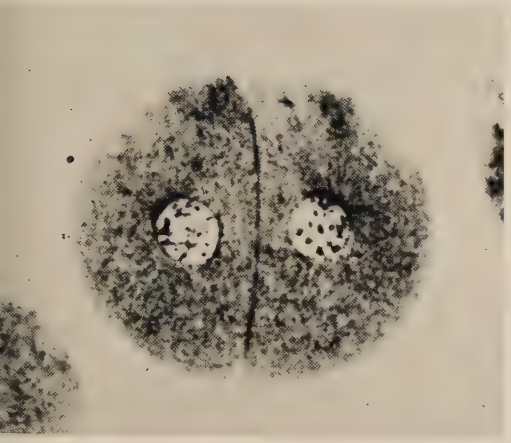

Fig. 5.

Fig. 6.

Fig. 7.

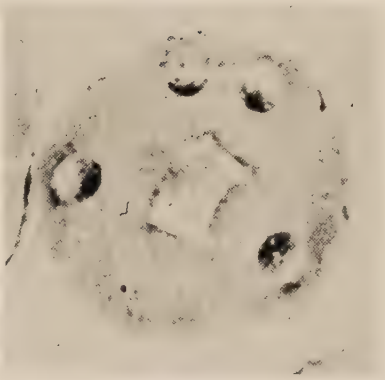

Fig. 8.

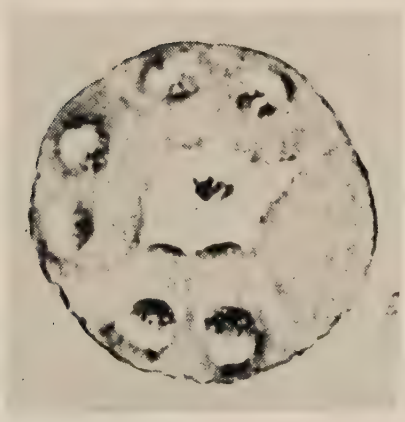

Fig. 9.

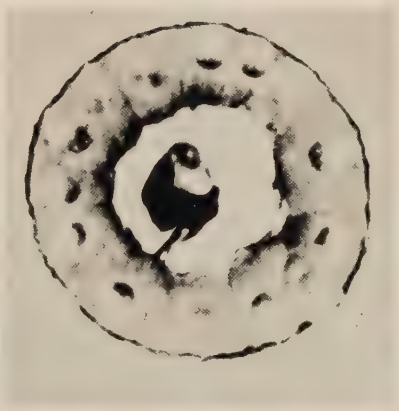

Fig. 10.

periodic acid and phenylhydrazin, as described above, but not upon treatment with hydrochloric acid as in the case of Feulgen staining. Thus, mucopolysaccharides are present, but thymonucleic acid seems to be absent from the resting nuclei of the cleaving eggs.

The alkalized (ammoniacal) silver nitrate solution is reduced by many polysaccharides in preparations pretreated with periodic acid, and by purines in preparations not subjected to the action of this oxidant. The cellular components become black if they are able to reduce this solution. The chromatin of the Feulgen negative nuclei appears colorless, and the chromatin of the Feulgen positive nuclei of the developing embryo appears black upon treatment with Gomori's alkalized silver nitrate solution in preparations not subjected to the action of oxidants. Thus, thymonucleic acid and purines contained in it are present in the Feulgen positive nuclei, and apparently absent from the Feulgen negative nuclei. This conclusion is also supported by the fact that the nuclei of the plutei and gastrulae become Feulgen negative and at the same time lose their ability to reduce the alkalized silver nitrate solution when deprived of nucleic acid by means of the procedure which has been recommended by Schneider (23).

The Feulgen negative and Feulgen positive nuclei of the sea urchin embryo are intensely stainable with many acid dyes, particularly with azocarmine G. This acidophilia disappears completely upon deamination (Monné and Slautterback, unpublished), but remains unchanged when nucleic acids are removed by means of the above mentioned procedure (Monné and Hårde, unpublished). This fact proves that the acidophilia is not due to some component of the nucleic acid (purine), but to the basic protein (histone) present within the chromatin. The Feulgen negative nuclei do not exhibit any affinity to toluidine blue, pyronin and some other basic dyes. This means that these nuclei are deprived of both ribonucleic acid and mucopolysaccharides with free sulfuric acid residues. Some hematoxylins, particularly iron hematoxylin, seem to be the only basic dyes which are able to stain the chromatin of the Feulgen negative nuclei. This fact may indicate that phosphoric acid is still associated with the chromatin which is deprived of nucleic acids. This conclusion is supported by the known fact that in histological preparations iron hematoxylin is able to react with phosphoric acid, but not with sulfuric acid residues.

Nevertheless, polysaccharides must be present because the nuclei of the unfertilized and cleaving eggs are intensely stained with fuchsin sulfite when the method described in this paper is applied. The chromatin of these nuclei does not show any distinct affinity to fuchsin sulfite. The chromatin is not stained,

but a substance coagulated in the interstices between the chromatin granules or precipitated on the surface of these granules. No doubt, a mucoid or mucolipid jelly fills the interstices between the chromosomes of the resting nucleus, as it was earlier supposed by Monné (16). This jelly is highly hydrated and therefore it cannot be fixed without being transformed into irregular coagula. Nevertheless, the coagula can be distinguished from the chromatin much more easily than expected because the substance stainable with fuchsin-sulfite is always present only in the one half of the nuclei (Figs. 6, 7) while the chromatin is either equally distributed throughout the whole nucleus (Fig. 5) or condensed in the other half of the nucleus. Moreover, the substance stainable with fuchsin-sulfite does not show any affinity to the dyes which are able to stain the chromatin of the Feulgen negative nuclei and in general to all the dyes which were mentioned above. It follows from this fact that the nuclear mucoid does not contain free amino groups or free sulfuric acid residues. It is also improbable that it contains free carboxyl groups (uronic acids). Thus, the mucoid present in the nuclei of the cleaving eggs seems to be neutral. Up to this time polysaccharides could not be detected within the nuclei of the oocytes by means of these staining methods. Nevertheless, the nucleoli were distinctly, although not very strongly, stained when oocytes fixed in Carnoy (not in Bouin) fluid were treated with periodic acid and fuchsin-sulfite. The central part of the nucleoli was more intensely stained than the periphery. It is possible that a detergent-like mucolipid is present within the nucleolus and that this nucleolus is dispersed during mitosis when the detergent-like mucolipid is activated upon increased hydration of its carbohydrate part.

As mentioned above only one half of the nuclei of the cleaving eggs is intensely stained with fuchsin-sulfite, always that which is directed towards the cleavage furrow or towards the blastocoele (Figs. 6, 10). Nevertheless, it is certain that the mucopolysaccharide is not completely absent from the other half of the nuclei. No doubt, there exists within the nucleus a gradient in the concentration of the mucopolysaccharide or mucolipid. More of this mucolipid appears to be present in the interior than in the exterior half of the nuclei. Thus, the nuclei exhibit a distinct polar organization. There exists a gradient in the concentration of the mucolipid and in the hydration of the nuclear substance. The interior halves are more hydrated than the exterior halves because more of the mucolipid is bound to the fibrillar texture of the former than to the fibrillar texture of the latter. Both halves differ distinctly in structure. The exterior half exhibits a denser structure than the interior one. The latter is more hydrated and therefore it is changed

more under the influence of the fixatives than the former. In the early blastulae the exterior part of the nuclei is somewhat flattened, which fact also proves that this part is less hydrated than the interior one. Nevertheless, the nuclei exhibit this shape only some time before mitosis and round up readily under the influence of fixatives.

The cytoplasm of the cells of the early blastula exhibits a distinct polar organization. The exterior cytoplasmic region adhering to the flattened part of the nucleus is more condensed than the interior one which adheres to the swollen part of the nucleus. A short time before mitosis the nucleus is elongated in a tangential direction, on the interphase between the more and less condensed cytoplasmic parts. This kind of cytoplasmic structure is responsible for the fact that the spindles are always oriented in a tangential direction and the division planes in a radial direction.

By means of the described method it is possible to distinguish at least four different polysaccharides. More exactly speaking it is possible to distinguish glycogen from three different mucopolysaccharides present within the sea urchin egg. There is no doubt that it will be possible to push the analysis still further when this method can be combined with the use of enzymes acting specifically on various polysaccharides.

The yolk of sea urchins consists of protein, lipid (16) and chiefly of a carbohydrate which appears to be an amino sugar. It may be that the yolk is a protein-amino-sugar-lipid compound. Various mucolipids which contain amino sugars, particularly the so called Forsman hapten are of widespread occurrence among animals and plants (1, 24). Mucolipids have been supposed to be polar-nonpolar compounds able to act like detergents (16). Amino-sugar-lipid compounds may act similarly to basic detergents. Commercial basic detergents are able to precipitate the jelly coat of the egg (15). It would be interesting to know whether or not the dispersed yolk substance is able to produce the same effect directly or with the aid of some enzyme. At any rate in injured eggs the yolk is rapidly liquefied and the jelly coat is precipitated. Cautious centrifugation of the eggs is sufficient to change the condition of the yolk. A granular fertilization membrane is frequently elevated upon fertilization of centrifuged eggs. The reaction between the basic substance of the yolk and the acid substance of the cortical granules may be responsible for this effect.

As mentioned above the mucoid of the Feulgen negative nuclei is neutral. It would be important to know whether or not the mucoids are different in different kinds of nuclei. At any rate some change in the properties of the nuclear mucoids occurs during maturation.

The ground cytoplasm exhibits a coarse structure in the mature sea urchin eggs and a fine structure in the fertilized ones (for details and literature see 22, 16, 17). It seems that the mucoid of the ground cytoplasm is provided with sulfuric acid residues (see p. 477). Moreover, it is probable that this mucoid is associated with lipids. Stained vacuoles formed within the sea urchin egg under the influence of neutral red or toluidine blue are artefacts. It is highly probable that vacuoles are formed when the mucolipids are separated from the cytoplasmic fibrils under the influence of the dyes. This conclusion is supported by the fact that the Golgi apparatus actually contains carbohydrates and that vacuoles are formed within this apparatus under the influence of neutral red. It was found that free lipid (19) and free carbohydrate (12) are bound to proteins upon fertilization of the sea urchin egg. Vasseur (27) advanced the view that this carbohydrate is not glycogen, but probably a mucopolysaccharide. Thus, it is highly probable that upon fertilization not free lipid and free mucopolysaccharide, but free mucolipid is bound to the cytoplasmic fibrils by means of its polysaccharide part. The binding of this detergent-like mucolipid (whose polysaccharide part is acid) may explain the fact that the hydration of the cytoplasm is increased, that the structure of this cytoplasm becomes fine and that stimulation is brought about upon fertilization. The Golgi-apparatus is dispersed and the structure of the cytoplasm becomes fine in strongly stimulated neurones (for details and literature see 16, p. 120, 17). This phenomenon occurs probably because the "free" mucolipid of the Golgi apparatus is dispersed throughout the cell and bound to the cytoplasmic fibrils. The physical properties of the cytoplasm are different in different regions, and change regularly during the functioning of the cell. This is probably because the mucolipid (or protein-mucolipid compound) is separated from the cytoplasmic fibrils in some regions of the cell and bound to these fibrils in other regions. The cytoplasmic texture is condensed in the regions of the cell where the concentration of this mucolipid is low, and loosened in the regions where the concentration of this mucolipid is high. In this connection it is of interest to note that protein-mucopolysaccharide particles with a diameter of about 10 mμ have been recently isolated from several tissues (11).

Acid cytoplasm seems always to contain both ribonucleic acid and mucoids with sulfuric acid residues. Basic cytoplasm cannot contain any considerable amount of ribonucleic acid and mucoids with sulfuric acid residues, but possibly mucoids composed of amino sugars. It may be that detergent-like mucolipids containing acid polysaccharides are present in cytoplasms rich in ribonucleic acid, and detergent-like mucolipids containing amino

sugars, in cytoplasms deprived of ribonucleic acid. Thus, different mucolipids or mucopolysaccharides may be present in the cytoplasm of different cells.

SUMMARY

Different properties are exhibited by the polysaccharides which are present in different components of the sea urchin eggs and embryos. Some of these components are stained yellow and others are stained violet in preparations successively treated with periodic acid, Albert and Leblond phenylhydrazin and Schiff fuchsin-sulfite (see p. 479). Thus, the yolk is stained yellow with phenylhydrazin, and the hyaline layer, the blastocoelic jelly, the jelly coat, the Feulgen negative nuclei of the unfertilized and cleaving eggs, the division spindle, the cortical granules and the coarse cytoplasmic nodes of the mature eggs are stained violet with fuchsin sulfite. This differential staining occurs only when treatment with phenylhydrazin is interrupted at the right moment. The polysaccharides which remain uncombined with this reagent, react with the fuchsin-sulfite which follows it. Upon prolonged treatment all polysaccharides react with phenylhydrazin and subsequent staining with fuchsin-sulfite becomes impossible. Aminopolysaccharides are most probably present within the yolk; sulfate-containing mucopolysaccharides within the jelly coat, blastocoelic jelly, hyaline layer, cortical granules and ground cytoplasm; and neutral mucopolysaccharides within the nuclei of the mature unfertilized and cleaving eggs. The physiological importance of mucolipids is stressed.

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BRIEF NOTES

THE CYTOPLASM OF LIVING TISSUE MAST CELLS IN VISUAL PHASE-CONTRAST

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Cytological observations on stained sections of normal and other freeze-vacuum dehydrated tissue mast cells by means of the ordinary light microscope indicate that the heparin-containing metachromatic constituents are located in the cytoplasm between the so-called large mast granules (2). Differential centrifugation data and electron micrographs have further shown that this material may be provisionally regarded as a microsome structure; a particulate matter was thus reported with an elementary diameter below 100 Å (2). Preliminary to more detailed studies on the submicroscopic structure of normal mast cell cytoplasm a note will be presented on the findings made under the phase-contrast microscope.

Selected very thin areas of rat and mouse mesentery were mounted under a cover-slip together with a drop of Ringer according to the technique used by Smiles *et al.* (1, 3). The preparations were sealed with wax and immediately inspected at room temperature under the phase-contrast microscope (Messrs. Cooke, Troughton & Simms) using a Wratten filter No. 61. A suitable mast cell was rapidly selected and a series of photographs were taken in a few minutes using a Wratten filter No. 28. During the time required no visible shrinkage or distortion of cells could be observed. Additional pressure was not applied to the tissues except that of the single coverslip.

In all pictures the following cytoplasmic structures could be clearly distinguished: (*a*) Large spherical granules, (*b*) Diffusely scattered "intergranular" amorphous cytoplasmic material, and (*c*) Areas of small interspersed "clear" spaces, *e.g.* cytoplasmic volumes presenting maximum light transmission. The structures are illustrated in Fig. 1.

The large granules showed variations both in size (0.3 to about 1 micron) and optical density. The largest ones presented a densely black picture. Most of them were spherical in shape, and a few were to all appearance elliptical or even rod-shaped (cp. review by Michels). This was the only particulate matter to be seen which presented the characteristics of mitochondria. Due to the low power of resolution no structure could be discerned in the intergranular amorphous material. The interspersed spaces showed mostly quite irregular configuration, and were devoid of visible limitations except the amorphous material. Some were actually rounded and consequently simulating vacuoles, but bulging from the free cell surface of such "pseudo-vacuoles" was nowhere seen. No distinct cell membranes could be

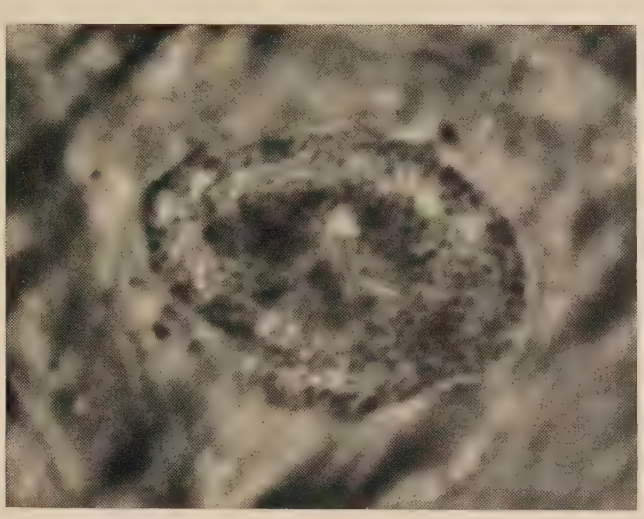

Fig. 1. Living mast cell from rat mesentery. Final magnification 2,450 ×. Oil immersion objective 1.8 mm, N. A. 1.30, Wratten filter No. 28. The "clear" spaces show a transmission comparable with that of the surrounding connective tissue. The nucleus is vaguely outlined in centre of cell.

discerned in these mast cells (cp. 4). Mast cell nuclei were faintly visible, but the nucleoli presented fairly good contrast.

It is easily understood that no attempts were made to apply staining reactions to such living mast cells.

There is a remarkably good agreement between the phase pictures just described and the ordinary microscopic pictures obtained in freeze-vacuum dehydrated specimens (2) as far as the large granules and the intergranular amorphous material is concerned. In the dehydrated cells, however, no discrete "clear spaces" have so far been noticed in this laboratory.

Previously published results (2) are consequently supported by phase microscopy, which method seems to provide a valuable tool also in this field of cytology.

ACKNOWLEDGEMENTS

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VITAMIN A AS A COMPONENT IN THE MECHANISM OF AEROBIC ENERGY TRANSPORT

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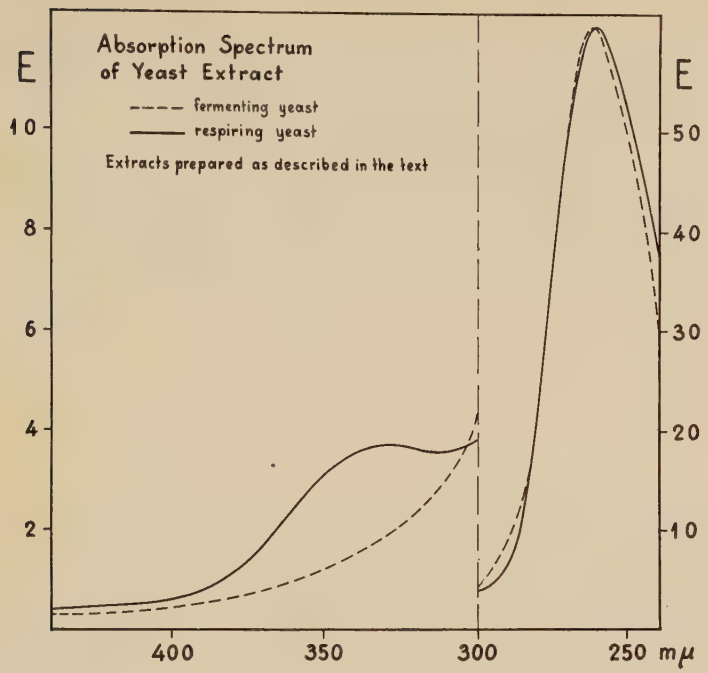
Vitamin A is, according to earlier investigations, included in the lipid phase of mitochondria (1, 3) and the theory has been stated that this vitamin has some correlation with the mechanism of oxidation. The oxygen uptake by liver in vitro, in the presence of Fe-containing porphyrines, rises with the addition of increasing amounts of vitamin A (2).

When isolating mitochondria from rabbit kidney with 0.25 M sucrose solution, according to Hogeboom, Schneider & Pallade (5), these authors made this solution 0.05 M with sodium fluoride. This procedure rendered possible the partial prevention of the decomposition of the highly labile phosphate bonds occurring in the adenylic acid complex of the mitochondria; similarly the more effective preservation of the amount of other coenzymes was achieved by the addition of the fluoride. A yellow extract with yellowish-green fluorescence was obtained on the extraction with 10 per cent trichloroacetic acid of mitochondria prepared in this manner. This extract contains vitamin A, as proved spectrophotometrically (absorption maximum at 325 mμ) and by the Carr-Price reaction as modified by Müller (6).

Since vitamin A can thus be obtained in a trichloroacetic acid extract and since it does not leave the water phase on shaking the extract with ether, it is suggested that vitamin A is linked — directly or indirectly — to a nonproteinlike hydrophil compound included in the water phase of mitochondria. All substances in this water phase are connected in their function with oxidative energy transport (4, 8). The fact that vitamin A participates in this enzyme system shows that it may be a component active in hydrogen transport.

The following experiment was made to prove this hypothesis. Since fermenting yeast is not considered to contain vitamin A, it was of interest to investigate whether the vitamin was active in yeast maintained under aerobic conditions.

1 kg of bakers' yeast suspended in 4 liters of water containing 50 g glucose, 50 ml ethanol, 5 g ammonium sulphate and 950 mg sec sodium phosphate was cultivated under a vigorous (2 kg) O_2-stream. The temperature was maintained between 25–30° C and the pH at 4.5–5.0 adjusted occasionally by addition of cocentrated ammonia. After two hours the yeast was centrifuged in the Sharpless Super Centrifuge (50,000 R. P. M.), suspended in 700 ml of peroxide-free ether and extracted in the Waring blendor by the addition of 700 ml ethenol, a clear, protein-free extract was obtained after centrifuging in the cold at moderate speed. Two phases were obtained on



adding to this extract a further 500 ml ether, the water phase being approximately 1/4 of the original volume. This water solution was washed three times with ether. The extracted lipids not strongly bound to hydrophilic compounds were thus removed. The remaining ether and a part of the ethanol were removed *in vacuo*. The clear solution thus obtained gave a positive Carr-Price test. A quantitative estimation gave 30 μg vitamin A/ml extract. As a point of reference it can be mentioned that an equal sample contained 200 μg inorganic phosphorus after twenty minutes' hydrolysis in N H_2SO_4.

Fig. 1 shows the absorption spectrum of extracts of fermenting and respiring yeast. The 329 mμ band of the respiring yeast is given by vitamin A which, in this preparation, seems to be present in the form of neovitamin A, a derivate isolated by Robeson & Baxter (7) from cod liver oil. This compound, which possesses the same biological activity as common vitamin A, has an absorption maximum at 328–329 mμ. The other maximum, at shorter ultraviolet wave length, is in both extracts due mainly to adenine. The extract of fermenting yeast has only this maximum.

The synthesis of vitamin A, for which carotines may be the precursors, begins, when the yeast, loses its fermenting power, because of the high oxygen pressure. This indicates that the vitamin A is necessary for, and involved in, the mechanism of aerobic energy transport. The occurrence of the vitamin in a water extract of mitochondria and respiring yeast shows that it is bound, in vivo, to a hydrophilic compound.

Further investigations are in progress to elucidate the structure and function of this compound which is essential for tissue respiration.

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ON THE INFLUENCE OF ATP ON THE FERTILIZATION AND SEGMENTATION OF THE SEA URCHIN EGG, STRONGYLOCENTROTUS LIVIDUS

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A NUMBER of observations from this laboratory have indicated that adenosine triphosphoric acid (ATP) has a pronounced effect on the cytoplasm of the sea urchin egg. The rigidity of the cytoplasm increased considerably upon the addition of 0.003 M ATP to an egg suspension in sea water (5). This was interpreted as being due to an increased linkage within gel-forming molecule complexes. Supplementary evidence resulting from other methods lent support to this view (5).

Aging of unfertilized eggs of *Arbacia lixula* in sea water for several hours often brings about a change in their response to insemination. The fertilization membrane is only incompletely differentiated, forming a gelatinous rim which remains attached to the cell surface (6). Wicklund found, however, that the egg retains its capacity of forming a normal membrane (4, p. 251) if 0.0002–0.001 M ATP is added to the sea water. The idea was entertained that the subnormal membrane differentiation observed in the Arbacia egg under certain conditions, may be due to an accumulation of an inhibitor in the surface of the egg. Further work demonstrated that the dissolved substance of the jelly coat which normally surrounds the egg, inhibits the membrane differentiation without suppressing the fertilization. The membrane appears in the form of the gelatinous rim mentioned above. Wicklund's results with ATP encouraged an attempt to test whether this substance was able also to counteract the inhibitory effect of the dissolved jelly.

The eggs of *Strongylocentrotus droebachiensis* obtained in March at the Kristineberg Zoological Station (at the west coast of Sweden) proved to be sensitive to the dissolved jelly. In the presence of 0.014–0.027 per cent of this substance in sea water, the membrane appeared upon insemination as a delicate layer attached to the egg surface. Upon transfer of the eggs to a hypertonic medium the membrane did not separate but adhered to the shrinking egg surface.

The addition of a sufficient quantity of ATP[1] to the jelly solution removes the inhibitory effect of this agent. The jelly solution seems primarily to inhibit the contraction of the egg which occurs upon the fixation of the spermatozoa to the egg

[1] A preparation from Sigma Chemical Co. was used. The Ba-salt was converted to Na-salt. Purity about 90 per cent.

TABLE I

The combined effect of jelly solution and adenosine triphosphoric acid on the membrane formation, the first segmentation and the further development in the egg of *Strongylocentrotus droebachiensis*. Temp. 5° C.

	Percentage of membrane elevation accompanied by contraction of the egg	Percentage first cleavage after 6 hours	Percentage further development after 18 hours
I. Control in sea water	100	90	100
II. Jelly solution 0.014 per cent	2—3	20	20
III. Jelly solution 0.014 per cent + 0.005 M ATP.....	60	90	80
IV. Jelly solution 0.027 per cent	0	20	30
V. Jelly solution 0.027 per cent + 0.005 M ATP.....	0	30	70

surface.[1] The contraction seems mainly to involve a flattening of one region of the egg surface with a corresponding bulging of other areas. The contraction thus takes place without no or only a slight change (2) in volume. In general the flattened part of the egg surface is adjacent to the entrance site of the spermatozoon. According to evidence earlier presented the contraction is instrumental in squeezing out from the egg a substance having a coagulating effect on the cortical components of the fertilization membrane (6). The effect of the jelly solution on the coagulation of the membranes is thus mainly an indirect one, although a certain direct effect cannot be excluded.

After addition of ATP together with the jelly, a membrane elevation takes place accompanied by a contraction of the egg — a process particularly conspicuous in eggs of *Strongylocentrotus droebachiensis*. Table I gives the result of one experiment. As indicated by the table, the addition of 0.005 M ATP solution eliminates to a great extent the inhibitory action of a 0.014 per cent jelly solution. It is not able, however, to counterbalance a 0.027 per cent jelly solution, but a pronounced improvement is found in this case as well, if the development to a late cleavage stage is considered. This result possibly implies that ATP is not only acting in the process of fertilization, but plays a part in later cleavage also.

Heparin (Na-salt, 80 int. units per mg)[2] 0.3–0.5 per cent in sea water has an action similar to that of the jelly solution. The inhibition is somewhat less pronounced with heparin than with the jelly solution. The action of heparin is also counteracted by ATP. Both membrane formation, cleavage and further development were improved upon addition of ATP.

[1] In some experiments dissolved jelly coats from oocytes proved to have very weak inhibitory effect. This possibly means that the inhibitory action of the jelly coat arises only after the maturation divisions.

[2] A preparation of heparin was received from the Vitrum factory by the courtesy of Professor Jorpes.

Eggs fertilized in sea water with 0.003–0.005 M ATP show a stronger contraction upon fertilization than eggs in pure sea water. An improvement of the membrane differentiation occurred often in the former case. Even when a well elevated smooth fertilization membrane appeared, the first division failed to occur or remained incomplete in the control. The eggs rather frequently showed this peculiar subnormal behavior the cause of which is unknown. In the presence of ATP 0.003–0.01 M, however, 100 per cent complete division occurred. In one experiment, for example, 40 per cent of the control eggs were in the stage of first cleavage after 6 hours (temp. 5° C). The eggs were elongated in one direction. The cleavage furrow was incomplete and often one-sided. Conversely, 100 per cent of the eggs in the samples with ATP (0.003, 0.005, 0.01 M) were in a two cell stage with complete separation of the blastomeres. Also in the following development remarkable differences were encountered. The control eggs cleaved into cells of uniform or somewhat varying size. In the latter case, however, no regular distribution of the cells with respect to the egg axis occurred. In the presence of ATP a high percentage of the eggs exhibited a regular cleavage pattern with the mesomere, macromere and micromere rings well differentiated.

The differences in cleavage pattern were reflected also in the early blastula stage. The cells were irregularly arranged in the control, whereas in the presence of ATP a regularly formed blastula wall was present.

It is known that a number of dyes, methylene blue, brilliant cresyl blue and dinitrophenol increase the respiration of sea urchin eggs but retard or inhibit the segmentation (1, 7). The results obtained by Loomis and Lipmann (3) demonstrate that the inhibitory effect of these substances is due to a dissociation of the hydrogen transfer reaction from the generation of phosphate bonds. From these data it is apparent that the generation of these bonds form a necessary link in the cell division mechanism.

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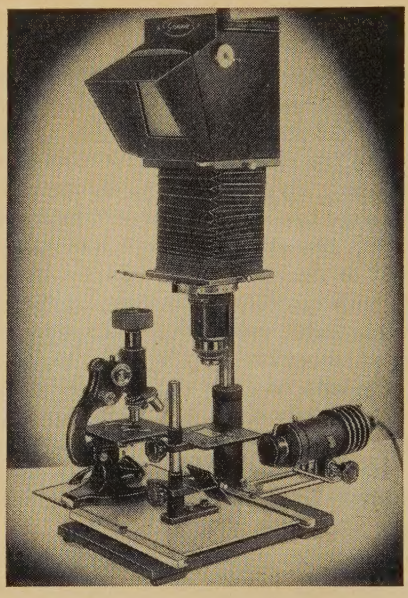

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